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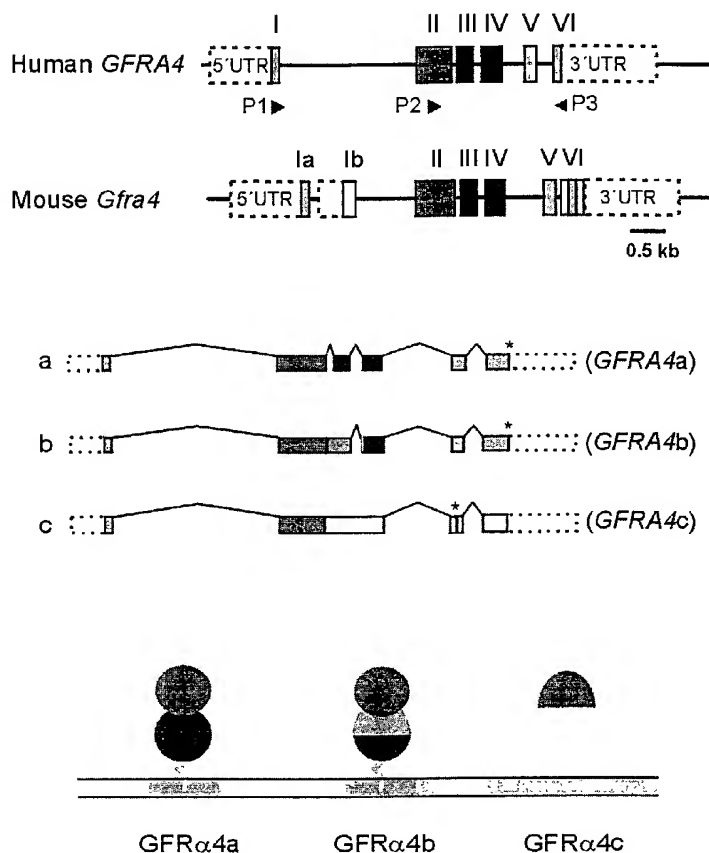
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(54) Title: COMPOUNDS RELATED TO OR DERIVED FROM GFR α 4 AND THEIR USE



(57) Abstract: The present invention discloses purified and isolated nucleic acid sequences encoding polypeptides having a structure substantially similar to that of splicing forms of mammalian GFR α 4 comprising the amino acid sequence (SEQ ID NO:1:) - (SEQ ID NO:6:). The preferred sequences comprise cDNAs having the sequence (SEQ ID NO:7:) - (SEQ ID NO :13:). The present invention is also related to purified and isolated polypeptides comprising the amino acid sequence and or substantially similar splicing forms of mammalian GFR α 4. Furthermore, the invention is related to substances capable of specifically recognizing said polypeptides and including both antibodies and receptors. The active compounds of the present invention including cDNAs, polypeptides, binding substances, and antibodies. The invention is useful not only for producing cell-lines and/or transgenic non-human animal but also for diagnosing and treating neuronal disorders or endocrine tumours as well as other related diseases by enabling measurements of GFR α 4-mediated signalling in endocrine cells and neurons.



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COMPOUNDS RELATED TO OR DERIVED FROM GFR α 4 AND THEIR USE**The Technical Field of the Invention**

The present invention discloses compounds related to, derived from or interacting with mammalian GFR α 4, a glycosyl-phosphatidylinositol (GPI)-linked GDNF family α -receptor. The invention relates to the mammalian protein as such or derivatives (homologs) thereof, nucleic acid sequences encoding said protein or derivatives thereof as well as modulators or specific binding substances of said GFR α 4 or GFR α 4-related substances. The compounds are useful especially for diagnosing and treating neuronal or neurological disorders, endocrine cell related diseases as well as tumours by recording GFR α 4-mediated signalling on said cells. The GFR α 4 or GFR α 4-like molecules or compounds interacting with said compounds are also useful for screening mimetics potentially useful as drugs.

The Background of the Invention

The GDNF family of neurotrophic factors includes four members: glial cell line-derived neurotrophic factor (GDNF), neurturin, artemin, and persephin (PSPN). GDNF family ligands signal through receptors consisting of a GPI-linked GFR α subunit and the transmembrane receptor tyrosine kinase RET. In order to activate the transmembrane receptor tyrosine kinase Ret, each of the GDNF family neurotrophic factors binds preferentially to one of the glycosyl-phosphatidylinositol (GPI)-linked GDNF family α -receptors (GFR α 1-4) (Airaksinen et al., 1999). GFR α 4, the preferential receptor for persephin (PSPN), has so far been described only from chicken (Enokido et al., 1998; Thompson et al., 1998). GDNF signals via GFR α 1, neurturin via GFR α 2, artemin via GFR α 3, whereas the mammalian GFR α receptor for persephin (PSPN) and the biological role of GFR α 4 has so far remained unclear.

RET-mutations are known to be related with inherited cancer

syndrome (MEN2), characterized by medullary thyroid carcinoma (MTC) and with sporadic forms of MTC and pheochromocytoma, but it is not clear why different specific RET mutations associate with the different disease phenotypes, for example, why hyperparathyroidism and familial MTC associate with certain types of mutations whereas sporadic MTC is associated with other types of mutations. For example *Ret* is expressed in malignant thyroid C-cells and adrenal chromaffin cells but also in normal cells non-affected in MEN2, leaving the cause for the cell specificity of the MEN2 cancer syndrome unclear.

Studies of expression and splicing patterns with mouse and human GFR α 4, surprisingly provided a response to the previous unclarities.

The Summary of the Invention

The novel cDNA sequences encoding GFR α 4 and corresponding to the mammalian splicing forms of mRNA enable the manufacture of new useful tools for diagnosis and/or treatment of certain endocrine tumours and other diseases related to endocrine cells and/or neurons in mammals, especially humans as well as for identifying and producing new products mimicking the compounds of the present invention.

The present invention is related to an isolated and purified nucleic acid sequence, which is a cDNA encoding a splicing isoform of mammalian GFR α 4 having the amino acid sequence (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), which are mouse derived or (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:), which are human derived. More specifically the cDNA sequences encoding human GFR α 4 molecules are characterized by comprising the nucleic acid sequence (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:). The genomic sequence (SEQ ID NO:13:) and fragments thereof including at least one of the exons (SEQ ID NO:29:), (SEQ ID NO:30:), (SEQ ID NO:31:), (SEQ ID NO:33:), (SEQ ID NO:35:), (SEQ ID NO:37:)

or (SEQ ID NO:39:) or any combination thereof with or without introns or parts of the introns (SEQ ID NO:32:), (SEQ ID NO:34:), (SEQ ID NO:36:) or (SEQ ID NO:38:) still encoding a polypeptide having a functional activity substantially similar to that of human GFR α 4.

The present invention is also related to the use of said nucleic acid sequence according to claims 1-5 for recording GFR α 4-mediated signalling in neurons or endocrine cells.

The present invention is related to the use of nucleic acid sequence according to claim 1-3 for manufacturing polypeptides useful for diagnosing and/or treating tumours in parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma and pheochromocytoma or for treating neuronal or neurological disorders.

The present invention is also related to purified and/or isolated polypeptides having the amino acid sequence (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) or other peptides, which have a structure substantially similar to an isoform of mouse GFR α 4 or of human GFR α 4.

The present invention is related to the use of said polypeptides for determining GFR α 4-mediated signalling in neurons or endocrine cells and for the use of said polypeptides for treating disorders and disease in the endocrine cells, such as thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells or cells of pituitary intermediate lobe, such as medullary thyroid carcinoma including pheochromocytoma and parathyroid hyperplasia, as well as neuronal disorders.

The present invention also include the use of said polypeptides for manufacturing a composition for diagnosing

and/or treating neuronal or neuronc disorders, osteoporosis, cancer in parathyroid gland cells, adrenal chromaffin cells and cells of pituitary intermediate lobe, in neoplasia, including treatment of endocrine tumours, including medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia.

The nucleic acid sequences and polypeptides of the present invention are also useful for recognizing substances which are mimetics of said sequences and are potentially useful as medicines for the diseases mentioned above.

Naturally the present invention is also related to methods for obtaining a cell-line (clone) useful in the production of cells, cell-lines and transgenic non-human animal.

The characteristics of the present invention are as defined in the claims.

Deposition:

The sequences reported in this paper have been deposited in the GenBank database and the accession numbers are listed below. Mus: AJ276870, AJ276871, AJ276872, AJ276514, AJ276515, AJ276516. Human: AJ291673, AJ291674, AJ291675

The Detailed Description of the Invention**Abbreviations**

ARTN, artemin; FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; GDNF, glial cell line -derived neurotrophic factor; GFR, GDNF family receptor; MEN, multiple endocrine neoplasia; MTC, medullary thyroid carcinoma; NRTN, neurturin; PSPN, persephin; PTC, papillary thyroid carcinoma, SCG, superior cervical ganglion

Definitions

In the present invention the terms used have the meaning they

generally have in the fields of biochemistry, pharmacology, recombinant DNA technology, including production of transgenic animal but some terms are used with a somewhat deviating or broader meaning than in the normal context. Accordingly, in order to avoid uncertainty caused by terms with unclear meaning some of the terms used in this specification and in the claims are defined in more detail below.

As used herein, **"a neurotrophic factor"** refers to a protein that modulates a biological activity of a cell, particularly neurons, but also other cells, through a neurotrophic factor signalling pathway, such as the RET signalling pathway, serine/threonine kinase Akt-mediated pathway or the RAS/MEK/MAPK pathway. Examples of biological activities in the present invention include, but are not limited to persephin.

As used herein, **"RET signalling pathway"** includes a cell, e.g., neural cell signalling pathway which involves the tyrosine kinase receptor RET. Said pathway includes the GDNF, neurturin, artemin and persephin signalling pathways, which neurotrophic factors bind specific GFR α coreceptors, GFR α 1-GFR α 4, which in turn bind and activate RET. As used herein "neurotrophic factor signalling pathway" includes a ligand, e.g. persephin (PSPN) independent signalling by GFR α 4/RET or GFR α 4 without RET.

As used herein, **"a neurotrophic factor responsive cell"** includes a cell which has a biological activity that can be modulated (e.g., stimulated or inhibited) by a neurotrophic factor. Examples of such functions include mobilization of intracellular molecules, which participate in a signal transduction pathway, production or secretion of molecules, alteration in the structure of a cellular component, cell proliferation, cell migration, cell differentiation, and cell survival. Cells responsive to neurotrophic factors preferably express a neurotrophic factor receptor, e.g., a GFR α receptor, such as GFR α 4, and/or tyrosine kinase receptor, e.g., the

tyrosine kinase receptor RET. Examples of neurotrophic factor responsive cells of the present invention include neurons, endocrine cells, such as thyroid C-cells and cells in adrenal medulla expressing GFR α 4.

Depending on the type of cell, the response elicited by neurotrophic factors is different. For example, in neuronal cells, neurotrophic factors regulate neuron survival and neuronal function. Abnormal or aberrant activity of proteins involved in specific trophic signalling pathways, can lead to a variety of disorders, for example in the endocrine system, e.g., abnormal growth (dysplasia or hyperplasia) as well as abnormal development, differentiation or function of the cells.

Abnormal or aberrant activity of a GFR α 4-protein (or abnormal or aberrant nucleic acid expression of the nucleic acid encoding said protein in a neurotrophic signalling factor pathway, in the nervous system or the endocrine system includes the above mentioned disorders in cells expressing or misexpressing GFR α 4.

In addition, neurotrophic factors, as such or combined with GDNF, artemin and neurturin and the interaction with GFR α -receptors also promote proper development of a variety of cell types. For example, neurotrophic factor/receptor interactions promote development, differentiation or function of certain peripheral organs and cells of the nervous system, innervation associated with said organs, tissues or cells. Activity of GFR α 4 signalling pathway in endocrine systems can lead to disorders including tumours associated with cellular development of cells of these organs as shown in the present RET invention. For example, axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures may aggravate the disease process. Augmenting GFR α 4 type of signaling in such conditions could be useful by preventing neuronal death without producing aberrant sprouting.

The term "**GFR α 4**" in the present invention means mammalian GFR α 4 and/or GFR α 4-like or GFR α 4-derived protein molecules or polypeptides, i.e. soluble receptor analogues expressed by and being substantially homologous to GFR α 4 at amino acid level. Said GFR α 4-like or GFR α 4-derived molecules defined in the claims are obtainable by isolation from natural mammalian, e.g. human or murine sources as different splicing forms. Said splicing forms lack the first Cys-rich domain characteristic of other GFR α receptors or said domain is easily spliced off during transcription or translation. The GFR α 4-like molecules of the present invention can also be produced by synthetic, semisynthetic, enzymatic and other biochemical or chemical methods including recombinant DNA techniques.

The amino acid sequences of the splicing isoforms or variants of mammalian GFR α 4 are listed below:

(SEQ ID NO:1:)	murine GPI-anchored isoform a1	Fig 18B;
(SEQ ID NO:2:)	murine putative transmembrane isoform a2	Fig 19B;
(SEQ ID NO:3:)	murine secreted isoform a3/4	Fig 20B;
(SEQ ID NO:4:)	human GPI-anchored isoform a	Fig 21B;
(SEQ ID NO:5:)	human putative GPI-anchored isoform b	Fig 22B;
(SEQ ID NO:6:)	human putative soluble isoform c	Fig 23B.

Polypeptide fragments useful for identifying and isolating nucleotide sequences encoding the above amino acid sequences are (SEQ ID NO:14:), (SEQ ID NO:15:), (SEQ ID NO:16:) and (SEQ ID NO:17:).

The "**GFR α 4-like or GFR α 4-derived molecules**" are substantially homologous with the amino acid sequences of mouse origin (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:) as well as of human origin (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:). They

all lack the first Cys-rich domain characteristic of other GFR α receptors and chicken GFR α 4. The "GFR α 4-like or GFR α 4-derived molecules" are characterized by comprising polypeptides such as the amino acid sequences (SEQ ID NO:14:), (SEQ ID NO:15:), (SEQ ID NO:16:) and/or (SEQ ID NO:17:) or amino acid sequences being truncated at the N-terminal or C-terminal end of the murine or human amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), (SEQ ID NO:4:), (SEQ ID NO:5:), (SEQ ID NO:6:), but still covering the contiguous and/or overlapping regions of the mammalian sequences (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), (SEQ ID NO:4:), (SEQ ID NO:5:), (SEQ ID NO:6:).

The term **"substantially homologous"** at amino acid level means that the GFR α 4-like protein molecules have a significant similarity or identity of at least 80%, preferably 85 %, most preferably more than 90 % with proteins encoded by human genomic sequence GFR α 4 (SEQ ID NO:13:)

In the present invention **"homology"** is determined e.g. using BLAST analysis combined with database searches matching the above defined expressed sequence tags (ESTs) with known sequences in the data bases. Nucleotide and amino acid sequence comparisons are also performed with (BestFit), alignment (PileUp), and phylogenetic tree (GrowTree). Also used are as signal sequence and transmembrane hydrophobicity plots (PeptideStructure) with the SeqWeb program package (Genetics Computer Group, Inc.).

The term **"GFR α 4-like molecules and derivatives thereof"** comprise polypeptides having the structure, properties and functions characteristic of GFR α 4-like molecules. Thus, the term "GFR α 4-like molecules and derivatives thereof" includes GFR α 4-like molecules, wherein one or more amino acid residues are replaced by another amino acid residue. Also truncated, complexed or chemically substituted, forms of said GFR α 4-like molecules are included in the term **"GFR α 4-like molecules and**

derivatives thereof". Chemically substituted forms include for example, alkylated, esterified, etherified or amidized forms preferably with a low substitution degree. Especially, useful are substitutions with small molecules, such as methyl or ethyl and a low substitution degree, because said small molecules and/or low substitution degrees, while stabilizing and modifying the GFR α 4-molecules of the present invention, are not prone to change or disturb the properties and functions of the GFR α 4-like molecules. The truncated, complexed and/or substituted variants of said GFR α 4-like polypeptides can be produced by synthetic or semisynthetic methods, including enzymatic and recombinant DNA techniques. The only other prerequisite is that the derivatives still are substantially homologous with and have the properties and/or express the functions of the domains of GFR α 4 and can be used for screening compounds mimicking the binding substances of GFR α 4.

The isolated GFR α 4 molecules should preferably comprise amino acid sequences which are at least about 70-80% or more homologous to the murine and human amino acid sequences defined above and should be characterized by at least one but preferably several of the following activities:

- 1) it can interact with (e.g. bind to) a neurotrophic factor, e.g. PSPN.
- 2) it can interact (e.g. bind to) a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET;
- 3) it can modulate the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET; and
- 4) it can bind a neurotrophic factor and modulate a response in endocrine cells, e.g., thyroid C-cells

The term **"GFR α 4-like molecules"** otherwise covers all possible splice variants of GFR α 4 expressed by mammalian tissues possibly excluding certain rat sequences, which are the thyroid, especially thyroid C-cells, and parathyroid glands

and cells in adrenal medulla and the pituitary intermediate lobe, and possibly cells of the nervous system.

As a conclusion **"GFR α 4-like molecules"** in its broadest aspect in the present invention, covers not only GFR α 4-like molecules derived from nature, including their isoforms of different origin, but also synthetically, semisynthetically, enzymatically produced GFR α 4-like molecules including molecules produced by recombinant DNA techniques. Said GFR α 4-like molecules can be used either as separate entities or in any combinations.

The term **"isoform"** refers to the different forms of the same protein, which originate from different sources, e.g. different mammalian species, e.g. human and murine sources in the present case. In the present invention the term, thus, includes fragments, complexes and their derivatives. For example, GFR α 4-like molecules can be generated by the cleavage of the corresponding preproprotein. Different reactions, including different enzymatic and non-enzymatic reactions, proteolytic and non-proteolytic, are also capable of creating truncated, derivatized or complexed forms of the GFR α -molecules.

Preferably, all **"GFR α 4-like molecules and their derivatives"** should be recognizable using binding substances capable of recognizing the native mammalian, including human and murine GFR α 4 or GFR α 4-like molecules. Such binding substances are for example the neurotrophic factor PSPN and/or fragments thereof and antibodies specifically recognizing GFR α 4.

In the present invention the term **"nucleic acid sequence"** means any isolated and purified nucleic acid sequences encoding mammalian GFR α 4 and/or GFR α 4-like substances or nucleic acid sequences with substantial similarity still encoding GFR α 4-like molecules having an amino acid sequence substantially homologous with the murine sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) and/or the human

sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:), lacking the first Cys-rich domain characteristic of other GFR α receptors (GFR α 1, GFR α 2, GFR α 3). Preferably the nucleic acid sequence should encode or express a polypeptide having the characteristics described above and at least one contiguous amino acid sequence (SEQ ID NO:14:), (SEQ ID NO:15:), (SEQ ID NO:16:) and/or (SEQ ID NO:17:).

The nucleic acids sequences of the present invention belong to the functionally active GFR α 4-related compounds of the present invention and they can be used as such or they can be introduced into suitable transformation or expression vectors, which in turn can be introduced into suitable host organisms to provide procaryotic, eukaryotic organisms capable of expressing altered levels of GFR α 4 as well as transgenic animals, e.g. knockout mice useful as test animals.

The "nucleic acid sequences" are selected from a group consisting of murine and human cDNAs listed below:

(SEQ ID NO:7:)	murine cDNA a1	Fig 18A
(SEQ ID NO:8:)	murine cDNA a2	Fig 19A
(SEQ ID NO:9:)	murine cDNA a3/4	Fig 20A
(SEQ ID NO:10:)	human cDNA (GPIa)	Fig 21A
(SEQ ID NO:11:)	human cDNA (GPIb)	Fig 22A
(SEQ ID NO:12:)	human cDNA c	Fig 23A
(SEQ ID NO:13:)	human genomic	Fig 24

Nucleotide fragments useful as probes or primers in hybridization or PCR-techniques and for identifying and synthetically preparing the human genomic sequence are (SEQ ID NO:18:), (SEQ ID NO:19:), (SEQ ID NO:20:), (SEQ ID NO:21:), (SEQ ID NO:22:), (SEQ ID NO:23:), (SEQ ID NO:24:), (SEQ ID NO:25:), (SEQ ID NO:26:), (SEQ ID NO:27:), (SEQ ID NO:28:), (SEQ ID NO:29:), (SEQ ID NO:30:), (SEQ ID NO:31:), (SEQ ID NO:33:), (SEQ ID NO:35:), (SEQ ID NO:37:), (SEQ ID NO:39:), (SEQ ID NO:40:). The introns of the human genomic

sequence include 5 introns, the first being an unsequenced about 500 bp long sequence situated between the nucleotides 97 and 98 in (SEQ ID NO:13:), (SEQ ID NO:41:), (SEQ ID NO:42:), (SEQ ID NO:43:), (SEQ ID NO:44:), (SEQ ID NO:45:), (SEQ ID NO:46:), (SEQ ID NO:47:), (SEQ ID NO:48:), (SEQ ID NO:49:) and/or (SEQ ID NO:50:). The second intron has the sequence (SEQ ID NO:32:), the third intron has the sequence (SEQ ID NO:34:), the forth intron has the sequence (SEQ ID NO:36:) and the fifth intron has the sequence (SEQ ID NO:38:). It is to be noted that the splicing variants may comprise introns or parts thereof.

The **"nucleic acid sequences"** of the present invention are not in their natural state but are isolated from their natural environment as transiently expressed mRNAs from different mammalian tissue. Thereafter, the mRNAs are purified and multiplied *in vitro* in order to provide by technical means new copies, which are capable of encoding said mammalian GFR α 4 or substantially homologous "GFR α 4-like molecules" of the present invention. Also the genomic sequence (SEQ ID NO:13:) and its complementary sequence are reproduced by copying the original strand or overlapping fragments thereof.

The isolated nucleic acid sequences of the present invention also include the human GFR α 4 encoding nucleic acid sequences SEQ ID NO:10:, SEQ ID NO:11: or SEQ ID NO:12:, which are a cDNAs identified by aid of the mRNAs from cDNA libraries from human thyroid and adrenal tissues as well as the genomic sequence (SEQ ID NO:13:) encoding and expressing a GFR α 4 protein or fragments thereof, which act as a receptor of the corresponding ligand, e.g. persephin.

The term **"genomic sequence"** means the corresponding sequence present in the nucleus of the mammalian cells and comprising introns as well as exons. In the present invention the genomic sequence is included as such and/or as separate exons or introns or combinations thereof. The splicing variants for

example may contain exons and part of introns as shown in the examples.

In the present context the term **"cDNA"** means a DNA sequence obtainable by reversed transcription of mRNA transcribed from the genomic DNA sequence. In the present invention the term cDNA also include the complementary strand of the original cDNA. Thus, nucleotide sequences hybridizing with the cDNA include both sequences substantially identical with the cDNA made as a copy of the respective mRNA but also the complementary strands and/or antisense strands. Genomic sequences copied are also included in the term cDNA.

The term **"nucleic acid sequence encoding or expressing GFR α 4-like molecules"** means nucleic acid sequences as well as substantially homologous nucleic acid sequences, which comprise at least one contiguous nucleic acid sequence selected from a group consisting of murine sequences (SEQ ID NO:7:), (SEQ ID NO:8:) or (SEQ ID NO:9:) as well as the human sequences (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:)

Among especially preferred nucleic acid sequences the contiguous sequences (SEQ ID NO:10:), (SEQ ID NO:11:) or (SEQ ID NO:12:) deduced from human cDNA can be mentioned. This sequence or their complementary sequences or nucleic acid sequences comprising parts thereof, e.g. fragments of said nucleotide sequences truncated at the 3'-terminal or 5'-terminal end, as well as sequences containing point mutations or SNPs caused by single nucleotide polymorphism are especially useful as probes for detecting nucleic acid sequences of the present invention.

Specific nucleic acid sequences useful as primers and/or probes are the nucleotide sequences (SEQ ID NO:18:), (SEQ ID NO:19:), (SEQ ID NO:20:), (SEQ ID NO:21:), (SEQ ID NO:22:), (SEQ ID NO:23:), (SEQ ID NO:24:), (SEQ ID NO:25:), (SEQ ID

NO:26:), (SEQ ID NO:27:) and/or (SEQ ID NO:28:), (SEQ ID NO:29:), (SEQ ID NO:30:), (SEQ ID NO:31:), (SEQ ID NO:33:), (SEQ ID NO:35:), (SEQ ID NO:37:), (SEQ ID NO:39:), (SEQ ID NO:40:), (SEQ ID NO:41:), (SEQ ID NO:42:), (SEQ ID NO:43:), (SEQ ID NO:44:), (SEQ ID NO:45:), (SEQ ID NO:46:), (SEQ ID NO:47:), (SEQ ID NO:48:), (SEQ ID NO:49:) or (SEQ ID NO:50:).

It is, however, clear for those skilled in the art that other nucleic acid sequence capable of encoding GFR α 4-like molecules and useful for their production can be prepared based on this information. The nucleic acid sequences encoding GFR α 4-like molecules should be capable of hybridizing under stringent condition varying e.g between 58°C, 2xSSC and 65°C, 0.1xSSC (Sambrook, J., et al., Molecular Cloning: A Laboratory Manual., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989) with sequence encoding the domain of the GFR α 4 or parts thereof.

The nucleic acid sequences of the present invention should have a substantial similarity with the SEQ ID NO:10:, SEQ ID NO:11: or SEQ ID NO:12. **"Substantial similarity"** in this context means that the nucleotide sequences fulfill the prerequisites defined above and have a significant similarity, i.e. a sequence identity of at least 60 %, preferably 70 %, most preferably more than 80 % with the nucleotide sequences encoding the murine amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) as well as the human amino acid sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:).

The term **"nucleic acid sequences encoding human GFR α 4 or GFR α 4-like molecules"** include their truncated or complexed forms as well as point mutations of said nucleic acid sequences as long as the GFR α 4-like molecules are capable of encoding amino acid sequences having the essential structural features as well as the properties and/or functions of GFR α 4-like molecules. The functions of the GFR α 4 or GFR α 4-like molecules or the compounds interacting with said

compounds can be measured with methods generally used for measuring the GDNF family receptor-GFR α mediated signalling including the method disclosed in the example(s) below.

The nucleic acid sequences are useful as such, for preparing transformation vectors and/or expression vectors as well as host cells carrying nucleic acid sequences capable of expressing the desired protein. They are capable of encoding/expressing GFR α 4 or GFR α 4-like proteins or fragments thereof. The GFR α 4 or GFR α 4-like molecules or compounds interacting with said molecules are useful for identifying or recognizing compounds mimicking said compounds, respectively. Binding substances are for example antibodies, receptors as well as shorter binding peptides, which specifically bind and recognize GFR α 4 or GFR α 4-like molecules or compounds interacting with said compounds. Said substances are useful especially for screening mimetics which may be useful in therapeutic or diagnostic applications. Such methods are well known to those skilled in the art. Examples of such methods it is referred to the following patent applications EP 639 584, WO 9638553, US 5,571,506, WO 200018790, WO 9950439, WO 9945930, US 6,030,619 and/or WO 9906599 which by reference are incorporated into the specification of the present invention.

The GFR α 4-like molecules can be identified and their amounts can be measured using **"binding substances"**. The term "binding substance" means any substances capable of specifically recognizing and binding the GFR α 4-like molecule or derivatives thereof or at least one specific portion of said GFR α 4 molecules. Such substances are, ligands of the GFR α 4 receptor or other binding proteins or peptides, comprising e.g. specific portions of the GDNF family neurotrophic factor, especially persephin (PSPN), capable of specifically binding GFR α 4-like molecules and taking part in the signalling specific for GFR α 4. Above all binding substances mean antibodies capable of specifically recognizing one or more GFR α 4-like molecules

alone or in any combination. The antibodies include both polyclonal and/or monoclonal antibodies as well as fragments thereof. Preferably, such binding substances recognize and bind to specific epitopes or active sites of the GFR α 4-like molecules.

Said binding substances can be produced using specific domains of GFR α 4 or any GFR α 4-like molecules, their isoforms as well as their fragments, derivatives or complexes with the prerequisite that they are capable of acting as "antigens". The antigens include any compositions or materials capable of eliciting an antibody response specific to said GFR α 4 or GFR α 4-like molecules or compounds interacting with said molecules. Ligands of GFR α 4-receptors, in other words specific neurotrophic factors are also included in the term binding substances. Said binding substances, preferably antibodies can be produced by conventional techniques for producing polyclonal antibodies as well as monoclonal antibodies. The methods for preparing monoclonal antibodies include hybridoma techniques. Fragments of antibodies or other binding proteins such as specific binding peptides can be developed by phage display techniques and produced by recombinant DNA techniques. All methods are well known by those skilled in the art and described in laboratory handbooks.

The term "**screening**" means that arrays of substances produced or recognized e.g. by combinatorial chemistry, with genomics or proteomics can be screened against the GFR α 4 compounds of the present invention or their binding substances in order to find compounds mimicking the properties or effects of said GFR α 4 or GFR α 4-like molecules and/or their binding substances are potentially useful as drugs or as models for developing further compounds. The results can be analyzed by methods provided by efficient tools in modern bioinformatics.

The term "**diagnosing**" means judging, predicting, assessing and/or evaluating as well as identifying and characterizing,

including screening, whether a person is susceptible of or suffers from endocrine cell related diseases or disorders in neurons, e.g. neurite outgrowth leading to neuronal death or aberrant sprouting. The diagnoses also enable evaluation of the severity of the condition, therapy required as well as the efficacy of treatment modalities or medical treatments needed. Especially, early identification of the disease, e.g. neuronal or neurological disorders in order to start prophylactic and/or other treatments before the onset of the actual disease is a desirable feature, enabled by the present invention.

The results are recordable with means for performing immunoassays using GFR α 4-like molecules and/or their binding substances as well as parts thereof. Means for performing amplification and hybridization methods using sequence specific probes or primers, which can be selected from the parts of the nucleic acid sequence fragments encoding suitable domains of mammalian, especially human GFR α 4.

The term "**screening**" may also mean the screening of a population for the presence or absence e.g. of autoantibodies. This screening means that based on the fact that people or subjects susceptible of or suffering from complications related to a certain said disease caused by GFR α 4 or GFR α 4-like molecules or compounds interacting with said compounds suffer from aberrant expression patterns which may elicit antibody production not occurring in subjects not suffering from the disorder. These so called autoantibodies can be determined using GFR α 4-like molecules. It is easy to screen a large population for the susceptibility of a certain diseases from blood or serum samples obtained from inflowing serum samples taken during routine diagnostic tests. If such autoantibodies can be detected in the serum of a person, it is a clear indication that the person in question is a potential patient and might be susceptible also to endocrine cell-related diseases or neurologic disorders and should be treated appropriately.

The term "**immunoassay**" refers to a immunochemical method or procedure capable of detecting and/or measuring at least one substance, either GFR α 4 or GFR α 4-like molecules or compounds interacting with said molecules or autoantibodies against said molecules using *per se* known means for performing an immunoassay, which means include a substance capable of specifically recognizing the substance to be determined, i.e. either at least one binding substance or a GFR α 4 or GFR α 4-like molecules or compounds interacting with said molecules or fragments thereof, for the desired application, respectively.

Well known examples of immunoassays are radioimmunoassays (RIA), radioimmunometric assays (IRMA), fluoroimmunometric assays (IFMA) enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), fluoroimmunoassays (FIA), luminescence immunoassays, immunoagglutination assays, turbidimetric immunoassays, nephelometric immunoassays, etc. All methods are well known by those skilled in the art and described in laboratory handbooks.

The preparation and development of methods and means for measuring different antigens and antibodies, which can be applied also to the determination of GFR α 4-like molecules and their autoantibodies have been described for example in the following patent publications applicable as convenient kits US 5,591,645, US 5,712,170, US 5,602,040, US 5,622,871, US 5,656,503, EP 149 168, US 4,552,839, US 4,361,537, US 4,373,932, WO 86/04683, EP 154 749, EP 7654, WO 86/03839, EP 191 640, EP 212 599, US 4,552,839, EP 158 746, EP 225 054 and which are herewith incorporated by reference. Even if said patents are restricted to the development of test kits for diagnosing other diseases by aid of binding substance recognizing the respective active molecule, the man skilled in the art can use the information for developing corresponding test kits for measuring GFR α 4-like molecules of the present invention.

Any immunochemical test methods can in principle be used for diagnosing endocrine diseases as well as for longitudinal or latitudinal screening of the progress of disease and effect of medical treatment. However, visual agglutination, flow-through and immunochromatographic methods are best suited for rapid assays or tests.

The term **"prophylactic treatment"** includes specific measures and/or precautions, e.g. control including possible medication, before the onset of disease. After the on-set of disease, prophylactic treatment requires therapeutic treatment as a precaution in order to avoid further complications.

The term **"therapeutic treatment"** includes methods for treating persons by administering a compositions comprising GFR α 4 or GFR α 4-like molecules or compounds interacting with said compounds, including GFR α 4-like polypeptides or GFR α 4-binding substances. Nucleic acid sequences encoding the same are useful in gene therapy or for preventing the genes causing the disease from expressing the gene products causing the diseases.

The term **"preparation or compositions"** means the active ingredients of the present invention including the nucleic acid molecules encoding GFR α 4, GFR α 4-like proteins and/or polypeptides, GFR α 4 modulators and antiGFR α 4 antibodies in combination with at least one pharmaceutically acceptable carrier, which is compatible with the active ingredient, i.e. GFR α 4 or GFR α 4-like molecules or compounds interacting with said compounds and the route of administration. The nucleic acid molecules of the present invention can be used in gene therapy by introducing the molecules to suitable vectors or other *per se* known delivery systems. The pharmaceutical compositions can be included in a container, pack or dispenser together with instructions for use.

The nucleic acid molecules, proteins, protein homologues, modulators and antibodies described can be used in drug-screening assay, diagnostic assays, methods of treatment, pharmacogenomics, proteonomics, bioinformatics and/or monitoring of effects during clinical trials.

The nucleic acids of the present invention can be used to provide mammalian vectors which in turn can be introduced into mammalian cells. Suitable expression systems for pro- and eukaryotic cells are described in Sambrook et al., 1989. The host cells can be used for expressing the GFR α 4-like proteins of the present invention.

The DNA constructs, vector construct and/or host cells of the present invention can also be used to produce non-human transgenic animals useful in screening assays designed to identify agents or compounds, drugs, pharmaceuticals, etc., which are capable of ameliorating detrimental systems related to specific neurons and/or endocrine cells. Especially useful are knockout mice capable of expressing altered levels of GFR α 4.

The molecules, including nucleic acid sequences, GFR α 4 or GFR α 4-like molecules interacting with said compounds and the products developed, characterized and/or produced based on said molecules or compounds of the present invention are useful for studying the role of GFR α 4-mediated signalling in neurons, in neoplasia, in endocrine cells and in thyroid calcitonin-producing C-cells (correlated with osteoporosis), parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma, parathyroid hyperplasia and association with specific mutations in the RET oncogene as well as in nervous system and testis.

The General Description of the Invention

The present invention is related to GFR α 4 or GFR α 4-like molecules or compounds interacting with said molecules which GFR α 4-molecules are members of the GDNF protein family including glial-cell derived neurotrophic factor (GDNF), neurturin, artemin and persephin signal through receptors consisting of GPI-linked GFR α subunits and the transmembrane tyrosine kinase Ret, but also other signalling pathways.

The present invention is based on preliminary studies made with mouse *Gfra4* which was characterized and shown to undergo developmentally regulated alternative splicing in several tissues. The results also indicated that mammalian GFR α 4 receptor lacked the first Cys-rich domain characteristic of the other GFR α receptors.

The glial cell line-derived neurotrophic factor (GDNF) family ligands GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN) are structurally related neurotrophic factors that signal through a multicomponent receptor composed of the transmembrane receptor tyrosine kinase RET and high affinity glycosyl-phosphatidylinositol (GPI)-anchored proteins, the GDNF family alpha receptors 1-4 reviewed by Airaksinen, et al., 1999 and Baloh, et al., 2000. GFR α 4 was first described from chicken and shown to be the preferential receptor for PSPN (Enokido, et al., 1998; Thompson, et al., 1998).

The mouse GFR α 4 receptor described herein was also disclosed by Lindahl, et al., 2000 after the filing of the priority application. It differs from all other GFR α receptors, including chicken GFR α 4 by being smaller in size and lacking the first Cys-rich domain. Mouse *Gfra4* transcripts were shown to be expressed in many embryonic and adult tissues but efficient splicing leading to a functional GPI-linked isoform, as well as putative transmembrane and soluble isoforms, occurred only in thyroid and adrenal medulla and in pituitary intermedi-

ate lobe. In mouse, *Gfra4* and *Ret* were co-expressed only in the thyroid C-cells and adrenal chromaffin cells. In chicken, *Gfra4* mRNA was broadly expressed during embryonic development, including the spinal motoneurons and kidney (Thompson, et al., 1998). Chicken *GFR α 4* has been shown to bind mouse PSPN and to confer a survival response to PSPN in the presence of *Ret* (Enokido, et al. (1998). However, due to different structures of chicken and mammalian *GFR α 4*, as well as the lack of information about the existence of chicken *GFR α 3*, ligand-specificity of mammalian *GFR α 4* cannot be directly extrapolated from experiments with chicken *GFR α 4*.

It is known that PSPN mRNA is expressed at low levels in many rat tissues, where two transcripts, an unspliced and a functional spliced form are produced (Milbrandt, et al., 1998; Jaszai, et al., 1998). PSPN is known to promote the survival of embryonic motor neurons *in vitro* and to rescue nigral dopamine neurons following neurotoxic injury *in vivo* but not to support the survival of any peripheral neurons tested (Milbrandt, et al., 1998). However, unlike GDNF and NRTN, PSPN does not induce neurite outgrowth of rat P8 motor axons in organotypic cultures (Ho, et al., 2000).

Somatic rearrangements were known to cause chromosomal inversions which activate the oncogenic potential of *RET* in human papillary thyroid carcinomas, whereas germline point mutations are known to be responsible of multiple endocrine neoplasia type 2 (MEN2; reviewed in Edery, et al., 1997; Eng, et al., (1999); Ponder, et al., 1999).

The malignancies associated with these syndromes are known to arise in several neural crest-derived cell populations. MEN2A is characterized by medullary thyroid carcinoma (MTC), pheochromocytomas and parathyroid hyperplasia, whereas MEN2B is characterized by MTC associated with pheochromocytomas, enteric ganglioneuromas, skeletal abnormalities and mucosal neuromas. MTC is the only phenotype in familial medullary

thyroid carcinoma (FMTC). In MEN2A, cysteine substitutions in the extracellular domain of RET cause aberrant homodimerization and ligand-independent constitutive activation of RET (Santoro, et al., 1995; Iwashita, et al., 1996), which alone or together with unknown factors cause the malignant proliferation of cells. FMTC mutations are more evenly distributed among the extracellular cysteines but are also found in the intracellular part of RET. It is known that most cases of MEN2B are caused by a specific mutation in the tyrosine kinase domain, which is also frequently found in sporadic MTC. MEN2B is caused by constitutive activation of RET with altered substrate specificity in downstream signalling pathway (Santoro, et al., 1995; Iwashita, et al., 1996).

However, in MEN2B-RET transfected cells, ligand binding has been reported to increase the intensity of RET signalling (Carlomagno, et al., 1998; Bongarzone, et al., 1998). It has been shown that transgenic mouse models of these mutations cause some of the malignant phenotypes found in human MEN2 syndrome (Michiels, et al., 1997; Smith-Hicks, et al., 2000). Phenotypic variability within the same MEN2 family and between different families carrying the same mutation in RET, suggests that further genetic events or modifier genes are required to induce the tumor phenotype in MTC.

The mouse *Gfra4* cDNA and genomic DNA sequence described in the present invention was characterized by having an unusual structure. The *Gfra4* pre-mRNA was alternatively spliced in tissue-specific manner that was developmentally regulated. A splice variant of *Gfra4* encoding GPI-linked isoform of GFR α 4 was selectively expressed in juvenile thyroid C-cells and parathyroid gland. In contrast, newborn and adult thyroid, parathyroid, pituitary and adrenal glands produced another *Gfra4* mRNA species, which encodes a putative transmembrane-anchored isoform. *Ret*-deficient mice were shown to have significantly less C-cells than their wild type

littermates suggesting that thyroid C-cells and possibly adrenal chromaffin cells require GFR α 4-Ret-signalling, which may specify tissues affected by the MEN2 cancer syndromes.

The nucleic acid sequences of the present invention encode an isoform of mouse or human GFR α 4 comprising the murine amino acid sequence (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) as well as the human amino acid sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:). Preferably, the sequences are cDNAs of murine origin having the sequence (SEQ ID NO:7:), (SEQ ID NO:8:) or (SEQ ID NO:9:) as well as the cDNAs of human origin (SEQ ID NO:10:), (SEQ ID NO:11:) or (SEQ ID NO:12:) encoding isoforms of mammalian GFR α 4. Also included among the nucleic acid sequences of the present invention are the genomic sequence of human GFR α 4 locus (SEQ ID NO:13:).

Originally, a murine GFR α 4 nucleic acid sequences was identified by the aid of available genomic sequences in formation of mouse mahogany gene locus (Gunn et al., 1999, Genbank AF155960). Within this genomic region, an "open reading frame" was identified that encoded a protein that showed sequence homology to chicken GFR α 4. Probes were generated based on portions of the genomic sequence and cDNA libraries were screened with said probes. Nucleotide sequences were determined and assembled and various methods such as RACE and genomic sequence analysis were used to extend the 5' sequence. Examples of the mammalian nucleotide sequences which were found and which encoded GFR α 4 were the mouse cDNAs (SEQ ID NO:7:), (SEQ ID NO:8:), (SEQ ID NO:9:) as well as the human cDNAs (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:) and the predicted amino acid-sequences were the mouse originated sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) as well as the human originated sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) also shown in Figures 18-24.

The present inventors also isolated and characterized the

human GFR α 4 receptor and showed that it is a functional co-receptor for PSPN, which mediates RET activation. Selective co-expression of GFRA4 and RET in normal and malignant C-cells and developing adrenal gland suggested that GFRA4 is a candidate modifier gene in medullary thyroid carcinoma and pheochromocytoma associated with the MEN syndromes.

The present invention is based on the amino acid and/or nucleic acid sequence defined above and in the claims, especially, the GFR α 4 cDNA, that encodes a specific member of the Glial Derived Neurotrophic Factor-Alpha Family of Receptors, i.e. the GFR α 4 protein, which functions in neurotrophic factor signalling pathways.

The first mammalian members of the GFR α 4 subfamily were identified, as described herein. Gfra4 was expressed in many mouse tissues including nervous system, where intron retention leads to a putative secreted GFR α 4 protein. Efficient splicing was shown to occur only in thyroid, parathyroid, pituitary, and less in adrenal glands. A splice form leading to a GPI-linked GFR α 4 receptor was shown to be expressed in juvenile thyroid as well as in parathyroid and pituitary glands.

Significant loss of thyroid C-cells in *Ret*-deficient mice suggested that C-cells, and cells in adrenal medulla also express *Ret* and may require signalling via GFR α 4-*Ret* receptor. Finally, GFR α 4 expression may restrict the inherited cancer syndrome multiple endocrine type 2, associated with mutations in RET, to these cells. These preliminary hypothesis were supported by further observations as disclosed below.

The first GFR α 4 proteins isolated were expected to bind to neurotrophic factors, especially GDNF-family ligands, and mediate signals within cells expressing the GFR α 4 protein. As expected, it was shown that the GFR α 4 protein transmitted a signal to the interior of the cell by activation of the RET

protein tyrosine kinase signalling pathway. Neurotrophic factors are known to promote survival and function of neural cells of both the central and peripheral nervous systems. Thus, modulation of the activity of a molecule involved in transmitting a neurotrophic factor signal to a cell results in modulation of the neurotrophic factor initiated cell function. Consequently, the ability of GFR α 4 to modulate neurotrophic factor initiated cell functions were tested. It was shown that GFR α 4 can be used to modulate neurotrophic factor action and/or activity and thereby GFR α 4 is useful for treating disorders associated with such functions (or lack thereof).

In addition, GFR α 4 mRNAs were expressed in a variety of tissues, especially, during embryogenesis, including, for example, cells of the pituitary gland and the intermediate lobe. Thus, modulators of GFR α 4 can be used to modulate development of these tissues and thereby to treat disorders associated with abnormal or aberrant development of these various tissues.

The molecular mechanisms by which the mutated Ret alleles contribute to the development of neuroendocrine neoplasms in MEN-2 syndromes is largely unknown. The effects of tyrosine kinase receptors are known to be mediated by the concerted activation of several signalling pathways including those of phospholipase C-, phosphatidylinositol 3-kinase, and the Ras/mitogen-activated protein (MAP) kinase (also known as ERK) (Kazlauskas, et al., 1994). In a rat pheochromocytoma cell line, PC12, activation of the Ras signalling cascade is a prerequisite for nerve growth factor (NGF)-induced cell differentiation (D' Arcangelo, et al., 1993).

Although Ras is implicated in Ret-induced neuronal differentiation, little is known about the contribution of this pathway to the biological effects triggered by Ret mutants in neuroectodermal cells (Rossel et al., 1997). For instance, the expression of active Ret mutants and their

causal function in neuroendocrine tumors, associated with MEN-2 syndromes, are difficult to reconcile with the dramatic differentiating effects observed when the same mutants are overexpressed either in PC12 or in human neuroblastoma cells (Borrello et al., 1995); Califano et al., 1995; Rossel et al., 1997). In PC12 cells, the Ret-induced molecular and morphological changes leading to differentiation are both mediated by Ras-dependent pathways (Califano et al., 2000). The studies of the present inventors using the claimed GFR α 4 molecules provided useful new information to this field.

It was generally believed that the serine/threonine kinase Akt-mediated pathway is responsible for most of the neurotrophin-regulated cell survival (for review see Kaplan and Miller, 2000). The evidence for the contribution of Ras/MEK/MAPK pathway to neuronal survival is rather conflicting. While neurotrophins induce a strong and sustained activation of MAPK in sympathetic neurons and PC12 cells, most studies have found that inhibition of MEK has minimal effects on neurotrophin-dependent neuronal survival. The major role for MAPK-induced survival pathways may be to protect neurons from death due to injury or toxicity, rather than from trophic factor withdrawal (Kaplan and Miller, 2000). Recent work of Ashcroft et al., 1999) has shown that while PI-3 kinase can contribute in part to neurite initiation processes, its selective activation and subsequent signalling to downstream effectors such as Akt, functions mainly to promote cell survival in PC12 cells. The Ras/MAPK pathway, on the other hand, is mainly responsible for neurite outgrowth and differentiation (Perron and Bixby, 1999), Ashcroft et al., 1999). Also in this respect the results of the present invention provided new information as regards GFR α 4s.

The transforming ability of several viral and cellular oncoproteins depends on their capacity to activate PI3-K.

a) Ret-MEN2A and MEN2B mediate enhanced PI3-K activity and

high phosphorylation state of its downstream signalling molecules;

b) Ret-mediated cell-transforming effect is known to be critically dependent on the activation of PI3-K/Akt pathway (Murakami et al., 1999; Segouffin-Cariou and Billaud 2000). In medullary thyroid carcinoma cell line TT, which carries Ret-MEN2A mutation, introduction of v-Ha-ras oncogene or activation of exogenous Raf (a kinase of MEK) leads to TT cell differentiation, a process opposite to oncogenic transformation (Nakagawa, et al., 1987; Carson-Walter, et al., 1998). Also as regards to these articles the present invention provides new useful data.

All members of GDNF family have shown to protect against excitotoxic motor neuron degeneration. In contrast, only GDNF and NRTN but not PSPN are able to induce neurite outgrowth in the organotypic culture of the same motoneuronal population. A specific inhibitor of MAP kinase, PD98059, inhibits the motor axon outgrowth-promoting activity of the GDNF but not the neuroprotective activity. (Ho, et al., 2000). Also as regards neurite outgrowth new data is provided by the present invention.

Because Ret *in vivo* is never expressed alone but always with a GFR α co-receptor (Golden, J. et al., 1999) it has been suggested that there is a difference in the activation of Ret signalling cascades depending on Ret interaction with one of the GFR α co-receptors. The signalling events might be different also when constitutively activated oncogenic Ret acts alone, without a co-receptor. This difference may lead to the predominance of either differentiating or surviving/proliferating cellular response. The present invention provides information suggesting that the Ras/MAPK pathway, being responsible mostly for cells differentiation in this paradigm, may undermine the action of PI3-K/Akt cascade, which can lead to the oncogenic transformation. The absence or inhibition of Ras/MAPK pathway on top of PI3-K/Akt activation

might be causative in the development of Ret-mediated MEN2 cancer syndrome, which is also shown below in the present invention.

The present inventor showed that unlabelled PSPN displaces [¹²⁵I] PSPN from GFRA4-transfected Neuro-2a cells, which express endogenous Ret. PSPN can be specifically cross-linked to mammalian GFR α 4 and Ret, and is able to promote autophosphorylation of Ret in GFRA4-transfected cells. PSPN, but not other GDNF family ligands, promotes the survival of cultured sympathetic neurons microinjected with GFRA4 and RET. Three different splice forms of human GFRA4 mRNA encoding for two GPI-linked and one putative soluble isoform identified by the present inventors were expressed exclusively in the thyroid gland. Overlapping expression of RET and GFRA4 but not other GFRA mRNAs in normal and malignant thyroid medullary cells suggests that GFR α 4 may restrict the MEN2 syndrome to these cells.

As already described above the present inventors in their preliminary studies identified from a putative mouse the *Gfra4* gene localized near the mahogany locus (Gunn et al., 1999) putative exons of the *Gfra4* gene homologous with other *Gfra* receptor genes and identical to several expressed sequence tags (see the Examples). The present inventors used RACE-cloning and RT-PCR of *Gfra4* cDNA from various tissues to identify the 5' and 3' sequences and splice variants of mouse *Gfra4* mRNA. All 5'RACE clones from the thyroid (including parathyroid) glands contained the signal sequence encoded by exon Ia (Fig 1A, 1C).

One 5'RACE clone contained an alternatively spliced exon Ib (Figure 1C), which would be translated into a weak putative signal sequence. All cDNA clones isolated from brain included a 53 bp intron between exons II and III, and the 5' extended up to 100 bp into the first intron upstream of exon II (Figure 1C). Translation of these mRNAs gave rise to a protein without

a signal sequence (not shown) (Figure 18, SEQ ID NO:1: and SEQ ID NO:7:).

The predicted amino-acid sequence of full-length mouse GFR α 4 from juvenile thyroid gland consisted of 263 amino-acids with a hydrophobic signal and C-terminal sequences typical for a putative GPI-anchored protein (Udenfriend and Kodukula, 1994). Mouse GFR α 4 showed higher amino-acid identity with chicken GFR α 4 (53%) than with chicken GFR α 1 (40%) and GFR α 2 (40%). However, the identity between mouse and chicken GFR α 4 was lower than the identity between mouse and chicken GFR α 1 (80%) or between the mouse and chicken GFR α 2 (75%). The alignment of mouse GFR α 1, GFR α 2, GFR α 3 and GFR α 4, as well as chicken GFR α 4 is shown Figure 1A.

The sequence of the GPI-anchored isoform of human GFR α 4 (Figures 21B and 22B, SEQ ID NO:4: or SEQ ID NO:5:) showed 79% amino-acid identity with the mouse GFR α 4 (SEQ ID NO:1:). Phylogenetic comparison indicated that the mouse GFR α 4 is more closely related to mouse GFR α 1 and GFR α 2 than to GFR α 3 (Figure 1B). Amino-acid sequence alignment (Figure 1A) showed that mouse GFR α 4 protein is smaller than the other GFR α -receptors. The mouse *Gfra4* gene (Figure 1C) contains at least six exons but it lacks the sequences corresponding to exons 2 and 3 of the other *Gfra* genes (Angrist, et al., 1998; Baloh, et al., 1998), which encode for the first Cys-rich domain (D1) and the hinge region in the putative GFR α domain structure (Airaksinen et al., 1999). Thus in contrast to other GFR α -receptors including chicken GFR α 4 that have three domains, mouse and human GFR α 4 has only two Cys-rich domains (Figure 1D).

Northern blot analysis of *Gfra4* expression in different tissues showed high mRNA levels in the developing and mature nervous system, thyroid gland and testis (Figure 2A). Moderate expression was seen in the adrenal gland and in several embryonic tissues (Figure 2A and not shown). The size of the

major *Gfra4* transcripts in testis (approx. 1.2 kb) shorter than in other tissues, such as 1-week-old brain (approx. 1.4kb). Longer transcripts (approx. 2.5 kb) were also present. (Figure 2A). *Gfra4* mRNA levels were low or not detectable in developing and adult kidney, muscle, spleen, liver, and salivary gland (Figure 2A and not shown).

RT-PCR was used to analyse *Gfra4* expression and to distinguish between the different splice forms in various tissues using primers spanning different exons. *Gfra4* mRNA comprising exon Ia was prominently expressed in the thyroid, parathyroid, and pituitary glands, and less in adrenal gland (Figure. 2B,C). The expression pattern of transcripts containing exon Ib was similar to those containing exon Ia. In testis and brain, in contrast transcripts containing exon Ia or Ib were barely detectable or undetectable, respectively (Figure 2B,C). This suggests that other exons encoding the N-terminal part of GFR α 4 protein are used in brain and testis.

Of all tissue examined, only thyroid, parathyroid and pituitary glands expressed major subset of *Gfra4* transcripts in which the short 53 bp intron separating exons II and III was correctly spliced (Figure 2B). Splicing of this intron occurred also in the adrenal gland but not in the brain or the testis. *Gfra4* transcripts with inclusion of the short 53 bp intron separating exons II and III were present in all tissues examined, including thyroid, pituitary and adrenal glands (Figures 2B and 2C). Translation of this intron causes a frame shift and premature stop codon producing a truncated protein with domain-2 (D2, Figure 1D) followed by a 53 amino-acid hydrophilic tail (SEQ ID NO:15:). This suggests that, regardless of possible signal sequence, testis and brain tissues examined would produce little, if any, membrane anchored GFR α 4. These results were confirmed by RNase protection assay using a probe that spans from exon II to exon IV (see Experimental Methods, data not shown). Translation of the transcripts that contain exon Ia and the intron (a3 and a4

in Figure 2C) would lead to production of a putative truncated isoform of GFR α 4 with 190 amino acids and strong signal sequence (SEQ ID NO:3:). In contrast, possible translation products of *Gfra4* transcripts that contain exon Ib and the intron (*b3* and *b4* in Figure 2C) might be poorly secreted.

Another alternative splicing event, which was shown to be developmentally regulated, involved the last exon (exon IV). A splice form that encodes the putative GPI-linked receptor was selectively expressed in the thyroid and parathyroid gland of juvenile 3-week-old animals (Figure 2B, transcripts a1 and b1 in Figure 2C.) Surprisingly, in newborn and 6-week-old mice, the thyroid and parathyroid glands preferably produced a longer splice form of *Gfra4* mRNA with an alternative usage of splice acceptor site in exon IV (Figure 2B, transcript a2 and b2 in Figure 2C). This exon IVb would translate into a putative transmembrane (TM) isoform of GFR α 4 (SEQ ID NO:2:) with and over 20 amino-acid hydrophobic stretch followed by a nine amino acid hydrophobic tail (SEQ ID NO:16:) instead of a putative GPI-anchor signal sequence (SEQ ID NO:17:) encoded by exon VIa. This TM splice variant was also expressed in adrenal and pituitary glands (Figure 2B). In conclusion, the splicing of *Gfra4* gene is both tissue-specific (with splicing of the short intron between exons II and III, and use of the signal sequence exon Ia) and also developmentally regulated (with alternative usage of different 5' splice sites of exon VI).

Splice site sequences of the short 53 bp intron are weak (Figure 3) with sub-optimal 3' polypyrimidine tract of the intron between exons V and VIa is also non-canonical. Weak splice sites are typically found at alternatively spliced sites, for example, in the calcitonin/CGRP gene that produces calcitonin in thyroid C-cells and CGRP in neuronal tissue. According to one model, additional RNA processing factors that help to recognize weak splicing sequences in calcitonin/CGRP gene are present in the C-cells (Lou and Gagel, 1998). It is possible that the tissue-specific and developmentally

regulated splicing of the *Gfra4* gene uses these same factors. However, although highly specific alternative splicing factors cannot be ruled out, the evidence found suggested the developmental and tissue specificity may arise from different ratios of general antagonistic and cooperating splicing factors as discussed by Lopez, 1998; Grabowski, 1998.

In situ hybridization was used to study the distribution of *Gfra4* and *Ret* mRNAs in mouse tissues (summarized in Table 1). *Gfra* was highly expressed in the intermediate lobe of developing (E14, E16 and P0) and mature pituitary gland (Figure 4A and Table 1), while *Ret* was expressed in the neurohypophysis and not in the intermediate lobe (Figure 4B). Moderate levels of *Gfra4* transcripts were found in several tissues at different embryonic and postnatal stages examined such as the condensing mesenchyme of developing bones (Figure 4C) and developing nervous system (Table 1). In adult spinal cord, *Gfra4* mRNA was present in most principal neurons including motor neurons (Figure 4D). In adult brain, *Gfra4* was expressed in neurons, with higher levels in the cerebral cortex and hippocampus than in the brainstem and cerebellum (Figure 4E). This expression pattern was strikingly different from that of *Ret* (Figure 4F). Moderate *Gfra4* expression was seen in the gut circular muscle and myenteric ganglia (Figure 4G,H), as well as in other peripheral ganglia, including the sensory dorsal root and trigeminal as well as superior cervical and sympathetic chain ganglia (Table 1). In testis, *Gfra4* mRNA was expressed in seminiferous tubules (Figures 4I and 4J). *Gfra4* and *Ret* were co-expressed in the neural crest-derived ultimobranchial body at E12 (Figures 5A-5C), E14, and also E16, when it starts to fuse with the thyroid diverticulum (Table 1).

In newborn mouse, highest expression of *Gfra4* was detected in distinct cells in thyroid medulla where calcitonin-producing C-cells are located (Figure 5D). Hybridization of adjacent sections with *Ret* probe (Figures 5E and 5F) showed a similar

pattern to *Gfra4*. Thus, the thyroid C-cells, which express *Ret* (Tsuzuki et al., 1995; Belluardo et al., 1999), co-express *Gfra4*. In contrast, other *Gfra* (*Gfra1-2*) mRNAs were not detected in the thyroid C-cells (data not shown) consistent with previous data) (Belluardo, et al., 1999; Golden, et al., 1999).

In postnatal thyroid gland, *Gfra4* (Figure 5G) and *Ret* (Figures 5H and 5I) co-expression continued in the C-cells. *Gfra4* mRNA levels remained abundant at least up to 17 weeks while *Ret* levels appeared to decrease (Table 1). Interestingly, the parathyroid gland, which expressed little if any *Gfra4* at birth (Figure 5D), expressed highly in 3- and 6-week old mice (Figure 5G and Table 1). However, we could not detect *Ret* expression in the parathyroid gland by *in situ* hybridization at any developmental stage examined (Figures 5E and 5I as well as Table 1).

Gfra4 and *Ret* were also expressed by newborn, postnatal and adult adrenal medulla (Figures 5J-5L and Table 1) at P8 while all cells in the medulla appeared to express *Gfra4* (Figure 5J), only a subpopulation of them, presumably chromaffin cells (Nakamura, et al., 1994; Tsuzuki, et al., 1995), contained *Ret* mRNA (Figure 5L).

The tissue-specific splicing, leading to putative GFR α 4 isoforms with either GPI- or transmembrane anchor, and co-expression of *Gfra4* and *Ret* in embryonic and postnatal thyroid C-cells suggested a physiological role for GFR α 4 in the development of these tissues. To test this possibility, we studied the number of C-cells in thyroid glands from *Ret*-deficient (-/-) mice. The number of calcitonin-immunoreactive cells in E18 *Ret* -/- mice appeared clearly reduced compared to control (wild-type or heterozygous) littermates (Figures 6A and 6B). Counting of immunoreactive profiles from serial reactions throughout the thyroid gland indicated a significant loss of about 37%

(control 2540 ± 240 , *Ret* -/- 1600 ± 100 , $n=4$ littermate pairs, $P<0.005$ using t-test). Thus, some thyroid C-cells seem to require *Ret*-signalling already during embryonic development. The selective expression of *Gfra4* in these cells suggests that $\text{GFR}\alpha 4$ mediates the *Ret*-signalling in C-cell development. Other C-cells may require $\text{GFR}\alpha 4$ /*Ret*-signalling postnatally, as *Gfra4* and *Ret* are co-expressed in these cells up to adulthood. The adrenal gland has been reported to be unaffected in newborn *Ret*-deficient mice (Durbec, et al., 1996). However, the expression of *Gfra4* and *Ret* in postnatal adrenal medulla suggests that signalling via $\text{GFR}\alpha 4$ -*Ret* receptor complex may be important in postnatal development of adrenal medullary cells. Finally, the expression of *Gfra4* and *Ret* in chromaffin and C-cells suggests that the putative ligand PSPN should have trophic or other biological effects on these cells.

Efficiently spliced *Gfra4* transcripts in mouse are expressed in thyroid, parathyroid and adrenal glands, that is, in tissues affected in humans with MEN2 syndromes, suggesting a causal link. GFRA4 is expressed and efficiently spliced also in human thyroid gland. In MEN2 cancer cells, deregulated RNA processing (Lou and Gagel, 1998) could lead to altered expression of *Gfra4* mRNA splice forms. $\text{GFR}\alpha 4$ -receptors may bind and modulate specific *Ret* mutants and could be essential for their oncogenic activity.

In the present invention the structure and expression patterns of human $\text{GFR}\alpha 4$ are described as well as the cloning of human $\text{GFR}\alpha 4$ cDNAs encoding three different isoforms of human $\text{GFR}\alpha 4$, two GPI-linked and one putative soluble isoform, from adult human thyroid gland. The cloning and expression of various mouse $\text{GFR}\alpha 4$ cDNAs encoding different protein isoforms have been described above. As in mouse, all human $\text{GFR}\alpha 4$ isoforms lack the first Cys-rich domain (D1) common for all known $\text{GFR}\alpha$ -receptors, including chicken $\text{GFR}\alpha 4$ (Thompson, et al., 1998).

Of 25 different adult and two fetal tissues studied, GFRA4 transcripts were detected only in the adult thyroid gland. However, it cannot be excluded that GFRA4 could be expressed in other tissues (such as pituitary intermediate lobe, which expresses high levels of *Gfra4* in mouse) or at earlier developmental stages (such as developing adrenal medulla) not analyzed here. However, this apparently exclusive expression of human GFRA4 in thyroid suggests a more tissue-specific transcriptional regulation and possibly a more restricted role for GFR α 4 in human, than in chicken and rodents, where *Gfra4* is expressed in many tissues (Thompson, et al., 1998). For example, the lack of GFRA4 in developing and adult human nervous system does not support a physiological role for PSPN as dopamine or motor neuron survival factor as was initially suggested using rat neurons (Milbrandt, et al., 1998).

Consistent with previous results disclosed by Nakamura, et al., 1994, Lorenzo, et al., 1995; Tsuzuki, et al., 1995) RET mRNA was expressed at variable levels in many adult human tissues, including the thyroid and adrenal glands. According to Milbrandt, et al., 1998 functional spliced PSPN mRNA is not present in humans. However, the present inventors found unspliced transcript of PSPN in all human tissues examined, whereas only low levels of spliced mRNA were detected in human adrenal gland, cerebellum, spinal cord and testis. In contrast, similar levels of both PSPN transcripts are present in most tissues in rat (Milbrandt, et al., 1998; Jaszai, et al., 1998). Although the spliced transcript encoding functional PSPN is expressed in the rat thyroid (not shown), its expression in either adult human (Figure 10) or postnatal mouse (not shown) thyroid gland could not be detected. Adrenal gland was the only tissue where the spliced PSPN transcript was detected in all three species analyzed (mouse, rat and human). The source of ligand for GFR α 4 in human thyroid remains elusive, although secretion of PSPN (from an unknown source) into the blood circulation can be speculated because

PSPN triggered Ret autophosphorylation in cells was detected only after serum deprivation (Poteriaev, D., unpublished data). It is also possible that functional PSPN is expressed in the thyroid region at earlier developmental stages not analyzed here.

Masure, et al., 2000 have reported a Gfra4 cDNA encoding a different GFR α 4 isoform. It was cloned from rat brain and reported as a GPI-linked receptor for PSPN. However, the signal sequence encoded by the rat Gfra4 cDNA in that report is very weak and differs from the GFR α 4 signal sequence disclosed in the present invention.

Sequence information from the preliminary research works using mouse Gfra4 cDNA and gene was used to identify human GFRA4 cDNA and genomic clones which is the main subject of the present invention. First, the genomic region covering putative exon 2 to exon 4 was cloned by PCR from human genomic DNA. Next, the structure of GFRA4 cDNAs and gene were characterized by 5' and 3'RACE of adult human thyroid cDNA and PCR of genomic DNA using primers corresponding to different regions of the putative exons of human GFRA4 as disclosed in the examples above. The sequence of human GFRA4 exon 1 encoding the signal sequence showed high homology to the mouse Gfra4 exon 1a. cDNAs encoding proteins with an alternative signal sequence homologous to the 1b signal sequence found in mouse, or putative transmembrane and soluble isoforms found in mouse, were not identified from human thyroid. Instead, three different alternatively spliced GFRA4 cDNAs were identified (GFRA4a, GFRA4b and GFRA4c, (SEQ ID NO:10), (SEQ ID NO:11) (SEQ ID NO:12), Figure 9B):

(i) GFRA4a (810 bp) corresponds to the mouse Gfra4 transcript a1. The predicted protein (GFR α 4a, 290 amino acids, Figure 9C) contains a putative N-terminal hydrophobic signal, one N-linked glycosylation site (NVSA) at position 178, and a hydrophobic stretch of amino acids in the C-terminus, preceded

by a hydrophilic linker region, consistent with a GPI-anchor signal sequence (Udenfried, et al., 1995). The amino acid identity between mouse and human GFR α 4a is 76%, whereas the identity between human and chicken GFR α 4 is 54% (covering amino acids 143-340 in chicken GFR α 4). The other alternative human GFR α 4 transcripts are:

(ii) GFRA4b (900 bp), in which the small intron (79 bp) separating exons 2 and 3 (Figure 9A) is included in the transcript, and the 3'splice site of exon 4 is located 11 bp upstream of the respective splice site used in the GFRA4a transcript. The small intron between exons 2 and 3 is also inefficiently spliced in majority of mouse tissues. Inclusion of this intron in mouse Gfra4 transcripts with exon 1a or exon 1b leads to putative soluble protein isoform. In contrast, translation of the transcript GFRA4b in human would lead to a GPI-linked protein of 299 amino acids. In this protein isoform, the N-and C-terminal ends are identical to the respective regions of GFRA4a, but the middle region consists of a stretch of 66 amino acids translated in different frame from intron 2 and exon 3. This sequence is not homologous to any protein in public databases.

(iii) GFRA4c (867 bp), in which the introns between exons 2 and 3, and between exons 3 and 4 are included in the transcript. These introns would lead to a frameshift with a stop codon located inside exon 5 and production of a putative soluble isoform of 236 amino acids. It is interesting to note that the sequences of the 3'splice sites of both mouse and human exon 3 and exon 4 contain a short polypyrimidine-tract interrupted with purines, which is characteristic to alternatively spliced exons (Lou, et al., 1994).

The present invention is also related to expression of GFRA4, PSPN and RET in different human tissues. Expression of GFRA4, PSPN and RET was analyzed by RT-PCR in 25 different adult and two fetal tissues (Figure 10). Expression of GFRA4 was only

detected in the adult thyroid gland (Figure 10) using primers P2 and P3 (see Figure 9). Similar results were obtained using different combinations of primers (data not shown). The smallest band of 699 bp corresponds to the GFRA4a cDNA where all introns are spliced out, whereas the 789 bp band corresponds to the intron 2-containing GFRA4b transcript. The 863 bp PCR fragment corresponds to the cDNA containing both introns 2 and 3 encoding the putative soluble isoform (GFRA4c, Figure 9B). The unspliced transcript (approx. 589 bp) of PSPN was expressed in all human tissues examined using primers detecting the full-length PSPN transcript (Figure 10, row 2). No transcripts were observed when RNA was used as a template for PCR, which shows that the cDNA samples did not contain chromosomal DNA. Low levels of spliced PSPN transcripts (476 bp) encoding the functional protein were present in human adrenal gland, cerebellum, spinal cord and testis, and were not detected in the thyroid gland. RET mRNA was expressed at variable levels in most tissues examined (Figure 10, row3).

The present invention is related to activation of human GFR α 4/Ret receptor complex, which is unique for PSPN. To study the binding of human PSPN to human GFR α 4, we used GFRA4a-transfected mouse neuroblastoma Neuro-2a cells (hGFR α 4/Neuro-2a), which endogenously express Ret. These cells strongly bound [125 I]PSPN, while mock-transfected Neuro-2a cells did not (Figure 11C). Low concentrations (Approx. 1 nM) of unlabeled PSPN effectively displaced [125 I]PSPN from the GFRA4-expressing cells, whereas GDNF (up to 300 nM), NRTN (up to 200 nM) and ARTN (up to 200 mM) were ineffective (Figure 11A). Thus, in the presence of Ret human GFR α 4 binds specifically PSPN, but not the other GDNF family ligands. PSPN binds to human GFR α 4 with IC₅₀ of 1 nM, and a dissociation constant (K_D) of approximately 100 pM (Figure 11A).

Next we studied the binding of PSPN to GFRA4a-transfected CHO cells, which do not express Ret. Treatment of the cells with [125 I]PSPN, followed by chemical cross-linking and analysis by

SDS-PAGE, resulted in a major band of approx. 46 kDa and minor bands of approx. 62 and 92 kDa (Figure 11B). The approx. 46 kDa band corresponds a PSPN monomer (approx. 14 kDa) cross-linked to monomeric GFR α 4 (30 kDa). The approximately 62 and 92 kDa bands most probably correspond to PSPN dimer cross-linked to GFR α 4 monomer and dimer, respectively. Additional 16 and 32 kDa bands represent PSPN monomer and dimer, respectively (Figure 11B, lane 6). No specific bands were detected from mock-transfected cells (Figure 11B, lanes 1 and 2). The amount of cell-bound complexes was greatly reduced by adding unlabeled PSPN or by removal of the GPI-anchored proteins by phosphoinositide-specific phospholipase C (PI-PLC) after cross-linking (Figure 11B, lane 4 and 5). Combination of these two treatments further reduced the yield of cross-linked products to undetectable levels (Figure 11B, lane 3). Thus, the human GFR α 4 protein, encoded by the GFRA4a transcript indeed contains a GPI anchor. Cross-linked complexes of the same sizes were also identified from hGFR α 4/Neuro-2a cells (Figure 11C). In addition, a minor band of about 200 kDa was observed (Figure 11C) that could be a complex of PSPN/GFR α 4 with Ret.

To study the interaction of PSPN with Ret, proteins cross-linked to [125 I]PSPN in hGFR α 4/Neuro-2a cells were precipitated with Ret antibodies (Figure 11D, lanes 2 and 4). The major cross-linked complexes of approximately 196 and 232 kDa, as well as minor complexes of about 62 and 92 kDa (same size as bands in Figure 11C), and also of approx. 400 kDa were obtained under reducing conditions (Figure 11D, lane 2). Under non-reducing conditions, the approx. 400 kDa band was greatly intensified, which indicates the presence of S-S-bound complexes. No cross-linked cell-bound products were detected using parental Neuro-2a cells (Figure 11D, lanes 1 and 3). The bands of about 196 and 232 kDa correspond to the complexes of PSPN/Ret (186 kDa) and PSPN/GFR α 4/Ret (216 kDa), respectively. The components of the complex of about 400 kDa could be a dimer of the 186 kDa PSPN/Ret complex.

To determine whether PSPN binding to human GFR α 4 mediates Ret autophosphorylation, hGFR α 4/Neuro-2a cells treated with PSPN were used. Gfra1 expressing Neuro-2a cells treated with GDNF served as a positive control. PSPN induced Ret tyrosine autophosphorylation in hGFR α 4/Neuro-2a cells, which showed a phosphorylated band of 170 kDa, corresponding to the active form of Ret (Figure 11E). No phosphorylation of Ret was observed in vector-transfected Neuro-2a cells (Figure 11E, mock). Stimulation of Ret phosphorylation was dose-dependent starting at 0.1 ng/ml of PSPN (Figure 11F).

The present invention is related to PSPN which specifically promotes survival of neurons ectopically expressing GFR α 4 and RET. In order to study whether binding of PSPN to mammalian GFR α 4/Ret complex triggers functional cellular programs, as described for chick GFR α 4 (Enokido, et al., 1998). The present inventors expressed the GPI-linked mouse Gfra4 or human GFRA4a together with human RET in neonatal mouse SCG neurons and maintained the neurons further with PSPN for three days. SCG neurons are trophically dependent on NGF, do not express full-length Gfra4 (not shown) and cannot be trophically supported by PSPN (Enokido, et al., 1998; Lindahl, et al., 1999; Milbrandt, et al., 1998). Significant portion of neurons expressing mouse GFR α 4 and RET was maintained by PSPN (60% versus 16% without PSPN, $p < 0.001$), whereas omission of GFR α 4 abolished this trophic effect (Figure 12). Similar results were obtained with human GFRA4a (not shown). Also GDNF and NRTN maintained part of neurons expressing RET and Gfra4, but these effects were not reduced by omission of Gfra4 (Figure 12). Moreover, both factors maintained also uninjected neurons similarly to receptor-injected neurons (Figure 12). ARTN promoted survival of neither GFR α 4/RET injected nor uninjected mouse SCG neurons (Figure 12). Thus, GFR α 4/RET complex requires PSPN, but not other GDNF family members, to activate a survival-promoting program in SCG neurons.

The present invention is related to selective expression of GFRA4 and RET mRNAs solely in medullary thyroid tumours. Co-localization of Gfra4 and Ret in developing and mature mouse thyroid C-cells and their co-expression in adult human thyroid tissue (Figure 10), prompted us to study the expression of GFRA4, RET and PSPN mRNAs in human primary thyroid tumors. RT-PCR analysis of GFRA4 expression, using primers generating full-length transcript, showed that similar levels of GFRA4 mRNAs encoding the two GPI-linked isoforms and lower levels of transcripts encoding the soluble isoform, are expressed in all eight MTC samples analyzed (Figure 13, row 1). GFRA4 mRNA was not detected in any of the follicular thyroid adenomas (FTA), follicular thyroid carcinomas (FTC) or papillary thyroid carcinomas (PTC) analyzed. Cellular localization of GFRA4 mRNA was studied by *in situ* hybridization in the same thyroid tumor samples (Figures 14A and 14B). GFRA4 was highly expressed by virtually all the malignant C-cells in the MTC samples analyzed, but not in the accompanying connective tissue and blood vessels. No expression of GFRA4 was detected in the adjacent apparently normal follicle cells found in some samples. Other thyroid tumors, including FTA, FTC and PTC did not express GFRA4. None of the thyroid tumors expressed detectable levels of spliced PSPN mRNA, whereas the unspliced form of PSPN transcript was present in all tumor samples analyzed (Figure 13, row 2). High levels of RET expression was seen in all MTC tumors (Figure 13, row 3 and Figure 14).

Tumor samples were further analyzed for GFRA1, GFRA2 and GFRA3 mRNA expression by RT-PCR and *in situ* hybridization (Figure 14A and Table 2). In some tumor samples, low levels of GFRA1, GFRA2 and GFRA3 mRNAs were present in subsets of the tumor cells. To study if GFRA4 gene is mutated in the MTC tumors, full-length GFRA4 cDNA clones from eight MTC samples, the TT cell-line, and normal thyroid were sequenced. Except for a few polymorphisms in the coding region, which do not change the amino acid composition of GFR α 4, no mutations were found in

the MTC samples or in the TT cell-line. Thus, the coding region of GFRA4 gene is not mutated in MTC tumors.

The present invention is related to a PSPN-GFR α 4-Ret complex inducing only the PI3-K/Akt pathway. To test the possible difference between GDNF-GFR α 1-Ret and PSPN-GFR α 4-Ret signalling mouse neuroblastoma Neuro-2a cell lines stable transfected with either GFR α 1 or hGFR α 4. Neuro-2a cells express endogenous Ret. Stimulation of Neuro-2a/GFR α 1 cells with GDNF lead to the activation of MAP (Figure 16A) as well as Akt kinases (Figure 15A). Stimulation of Neuro-2a/hGFR α 4 cells with PSPN also lead to the Akt activation (Figure 15B), however, the level of MAPK phosphorylation remained unchanged (Figure 16B). This difference between GDNF/GFR α 1 and PSPN/hGFR α 4 evoked responses appeared also at the physiological level. GDNF and PSPN, acting via their respective co-receptors, both were able to stimulate Neuro-2a cell survival in serum-free conditions (Figure 17A). In another assay, GDNF but not PSPN was able to induce the neurite outgrowth from the Neuro-2a cells transfected with either GFR α 1 or hGFR α 4, respectively (Figure 17B). These results suggest that while GDNF-GFR α 1-Ret system activates both Ras/MAPK and PI3-K/Akt cascades, the PSPN-GFR α 4-Ret complex can induce only the PI3-K/Akt pathway. PSPN-GFR α 4-Ret signalling may be unable to activate the MAPK activity or it may be suppressed during this signalling. Consequently, GFR α 1- and GFR α 4-specific pathways lead to either mainly differentiation or survival.

In the present invention it was also shown that unlabelled PSPN displaces [125 I] PSPN from GFRA4-transfected Neuro-2a cells, which express endogenous Ret. PSPN can be specifically cross-linked to mammalian GFR α 4 and Ret, and is able to promote autophosphorylation of Ret in GFRA4-transfected cells. Furthermore, it is shown that PSPN, but not other GDNF family ligands, promotes the survival of cultured sympathetic neurons microinjected with GFRA4 and RET.

In the present invention it has further been shown that GFR α 4 is the functional receptor for PSPN. The binding and cross-linking studies demonstrated that association of PSPN with the Ret receptor protein tyrosine kinase is mediated by GFR α 4. Ligand displacement binding showed that in the presence of Ret, only PSPN but not GDNF, NRTN or ARTN was effective in displacing [125 I]PSPN from GFRA4-expressing Neuro-2a cells. The dissociation constant (KD) of about 100 pM found here is ten times lower than the KD of about 1 nM reported for mouse PSPN binding to chicken GFR α 4 (Enokido, et al., 1998), and 60 times lower than the about 6 nM reported for rat PSPN binding to immobilized rat GFR α 4 fusion protein reported by Masure, et al., 2000, but similar to those reported for GFR α 1, GFR α 2 and GFR α 3 and their cognate ligands (Klein, et al., 1997; Trupp, et al., 1998; Baloh, et al., 2000). The lower binding of mammalian PSPN to chicken GFR α 4 probably reflects the species difference in GFR α 4 structure. PSPN has not yet been characterized from chicken and, if it exists, might differ significantly from the mammalian PSPN.

The cross-linking studies show that PSPN is able to bind GFR α 4 also in the absence of Ret. This is consistent with the model that a GDNF family ligand first binds to the corresponding GFR α receptor and subsequently the ligand-GFR α complex binds to Ret (Jing, et al., 1996). However, our results also agree with the alternative model in which the ligand binds a preformed GFR α /Ret complex (Sanicola, et al., 1997). Although PSPN binding to chicken GFR α 4 has been shown earlier (Enokido, et al., 1998).

In the present invention it is demonstrated for the first time that the association of PSPN with GFR α 4 results in activation of the Ret tyrosine kinase. PSPN treatment of cells expressing GFR α 4 rapidly induced Ret autophosphorylation in a dose-dependent manner. PSPN is unable to stimulate Ret autophosphorylation in cells that do not express GFR α 4. Thus,

the GFR α 4a isoform characterized in the present invention is a functional receptor for PSPN in triggering Ret activation. In contrast to this, the rat GFR α 4 isoform with a poor signal sequence described by Masure et al., 2000 bound PSPN only as soluble fusion protein and did not lead to Ret activation.

In the present invention it has also been demonstrated that PSPN but not other GDNF family ligands, can promote the survival of cultured SCG neurons ectopically expressing Gfra4 and RET. In our assay, neurons co-expressing RET plus GFR α 4 showed an elevated response to GDNF and NRTN but not to ARTN, however, also the uninjected neurons responded to GDNF and NRTN. Therefore, it seems that GDNF and NRTN maintain these neurons via activation of endogenous binding sites and not via GFR α 4. Taken together, the binding of PSPN to GFR α 4/Ret complex triggers functional cellular responses.

In the present invention the putative role for GFR α 4 in medullary thyroid carcinoma is demonstrated. As the RET proto-oncogene plays an important role in the oncogenesis of MTC, a logical step was to examine GFR α 4 expression in these tumors. GFRA4 transcripts encoding the two GPI-anchored isoforms were expressed at high level in all MTC samples, whereas no GFRA4 expression was detected in any other type of thyroid tumor analyzed. In addition, moderate levels of GFRA4c encoding the putative soluble form of GFR α 4 were found in all MTC samples. Taken together, the data provided by the present inventors suggest that MTC-specific expression of GFRA4 could have diagnostic value.

Strong RET expression was also localized to the malignant C-cells consistent with previous reports (Nakamura, et al., 1994; Santoro, et al., 1990). Low levels of GFRA1, GFRA2 and GFRA3 transcripts were present in subsets of tumor cells, indicating that their expression is not specific to particular thyroid tumor cell type. This result is in line with a recent report showing GFRA1 and GFRA2 expression in some MTC tumor

cells (Frisk, et al., 2000). However, our results showing low GFRA1 and GFRA2 expression also in normal thyroid, in other thyroid tumor types, and not in all MTC samples, does not support the idea that GFR α 1 or GFR α 2 play a role in the primary pathogenesis of MTC. Phenotypic variability within the same MEN2 family or between different families carrying the same mutation in RET suggest that further genetic events or modifier genes are required to induce the tumor phenotype in MTC. The results of the present invention indicate that co-expression of GFR α 4 with mutated RET may be necessary for the initial hyperplasia of C-cells occurring in MTC. It should be possible to test this hypothesis by examining whether the development of C-cell hyperplasia and subsequently MTC, pheochromocytoma and ganglioneuromas are suppressed in Gfra4-deficient mice crossed with MEN2 transgenic mice (Smith-Hicks, et al., 2000; Acton, et al., 2000; Laurikainen, et al., 2000).

Further studies with metastases derived from MTC tumors and other endocrine tumors such as GFR α 4 expressing pheochromocytomas provide more evidence as regards these questions. Further *in vitro* studies on cell lines and primary thyroid C-cells should also be informative to reveal the roles for PSPN-signalling via different GFR α 4 isoforms through normal and mutant RET.

Similar results were obtained from all thyroid tumours analyzed: GFRA4 was highly expressed in all tumour cells of 6 medullary carcinoma samples but not detectable in cells from other thyroid tumours including tissue samples from 4 follicular adenomas, 4 follicular carcinomas and 5 papillary carcinomas. samples. The six medullary carcinomas analyzed included both sporadic MTC, as well as tumors from patients with inherited MEN2 syndrome (carrying different mutations in RET).

As a conclusion the present invention is related to mammalian,

but especially human GFR α 4 and GFR α -like molecules and/or compounds interacting with said compounds, where GFR α 4 is the ligand-binding subunit required together with RET for PSPN signalling.

The invention provides isolated and purified nucleic acid sequences (e.g. cDNAs) comprising a nucleotide sequence encoding a GFR α 4 protein or a fragment thereof. Also described are fragments of said nucleotide sequences, which are suitable as primers or hybridization probes for the detection of GFR α 4-encoding nucleic acid sequences (e.g. mRNA). The isolated nucleic acid sequences encode the murine amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) and the human amino acid sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:), particularly the murine nucleotide sequences (SEQ ID NO:7), (SEQ ID NO:8:) or (SEQ ID NO:9:) and the human nucleotide sequences (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:), (SEQ ID NO:13:).

The isolated nucleic acid sequences of the present invention hybridize under stringent conditions to sequences which are at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 98% or more homologous to the nucleic acid sequences defined in the claims.

The GFR α 4 protein or polypeptide fragments or a biologically active portion thereof can be operatively linked to a non-GFR α 4 polypeptide to form a fusion protein.

The isolated and purified nucleic acid sequences encode proteins or fragments thereof including amino acid sequences which are sufficiently homologous to a protein comprising the murine amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) as well as the human amino acid sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) as long as the protein or the fragment thereof maintains the specific GFR α 4 activities described above. Preferably, the GFR α 4-protein or

fragments should maintain the ability to bind to a neurotrophic factor and modulate a cellular response in the way defined in the claims and as described above. The protein encoded by the nucleic acid sequences should be at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 98% or more homologous to the murine amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) or the human amino acid sequences (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) or the fragments (SEQ ID NO:14:), (SEQ ID NO:15:), (SEQ ID NO:16:) or (SEQ ID NO:17:).

The protein should preferably be a full length protein which is substantially homologous to the entire murine amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) or the entire human amino acid sequences (SEQ ID NO:4:), (SEQ ID NO:5:), (SEQ ID NO:6:) or the fragments (SEQ ID NO:14:), (SEQ ID NO:15:), (SEQ ID NO:16:) or (SEQ ID NO:17:) and includes all naturally occurring full length proteins and allelic variants and splice variants of human or murine GFR α 4.

The isolated GFR α 4s should preferably comprise amino acid sequences which are at least about 70-80% or more homologous to the murine and human amino acid sequences defined above and should be characterized by at least one but preferably several of the following activities as well as those described in the discussion of the experiments disclosed above:

- 1) it can interact with (e.g. bind to) a neurotrophic factor.
- 2) it can interact (e.g. bind to) a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET;
- 3) it can modulate the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET; and
- 4) it can bind a neurotrophic factor and modulate a response in endocrine cells, e.g., thyroid C-cells.

The isolated nucleic acid sequence should comprise at least 15-20 nucleotides capable of hybridizing under stringent

conditions to a nucleic acid sequence comprising the one of the murine nucleotide sequences (SEQ ID NO:7:), (SEQ ID NO:8:) or (SEQ ID NO:9:) or the human nucleotide sequences (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:) or parts thereof.

Preferably, the isolated and/or purified nucleic acid sequence corresponds to the naturally occurring nucleic acid sequence. More preferably, the isolated or purified nucleic acid sequences should encode naturally-occurring alleles and splice variants of human GFR α 4. Moreover, the GFR α 4-encoding murine cDNA sequences (SEQ ID NO:7:), (SEQ ID NO:8:) or (SEQ ID NO:9:) or the human cDNA sequences (SEQ ID NO:10:), (SEQ ID NO:11:) or (SEQ ID NO:12:) or (SEQ ID NO:13:) or cDNA fragments (SEQ ID NO:18:), (SEQ ID NO:19:), (SEQ ID NO:20:), (SEQ ID NO:21:), (SEQ ID NO:22:), (SEQ ID NO:23:), (SEQ ID NO:24:), (SEQ ID NO:25:), (SEQ ID NO:26:), (SEQ ID NO:27:), (SEQ ID NO:28:), (SEQ ID NO:29:), (SEQ ID NO:30:), (SEQ ID NO:31:), (SEQ ID NO:33:), (SEQ ID NO:35:), (SEQ ID NO:37:), (SEQ ID NO:39:), (SEQ ID NO:40:), (SEQ ID NO:41:), (SEQ ID NO:42:), (SEQ ID NO:43:), (SEQ ID NO:44:), (SEQ ID NO:45:), (SEQ ID NO:46:), (SEQ ID NO:47:), (SEQ ID NO:48:), (SEQ ID NO:48:) or (SEQ ID NO:50:), as well as strands which are complementary to the coding strand of the GFR α 4 cDNA sequences.

The invention is also related to recombinant transformation or expression vectors or constructs as well as procaryotic or eucaryotic, including mammal host cells comprising one or more of the nucleic acid sequences defined in the claims. Such host cells may be used to produce GFR α 4 proteins by culturing the host cell in a suitable medium. If desired, the GFR α 4-protein can then be isolated from the host cell.

The invention is also related to transgenic non-human animals in which a GFR α 4 gene has been introduced or altered. In such cases the genome of the non-human animal, e.g. a mouse, rat,

guinea pig, etc., can be altered by introduction of a nucleic acid sequences of the invention encoding GFR α 4 as a transgene. An endogenous GFR α 4 gene within the genome of the non-human animal can be altered or made functionally incompetent by homologous recombination or by using the knock-out technique.

The invention is further related to isolated GFR α 4 or GFR α 4-like molecules or compounds interacting with said molecules, e.g. biologically active fragments thereof. The isolated GFR α 4s or fragments can bind to a specific neurotrophic factor and stimulate a response in a neurotrophic factor responsive cell.

The invention also provides preparations or compositions of GFR α 4s which comprise amino acid sequences of the GFR α 4s defined above in combination with carriers or diluents which can be pharmaceutically acceptable or not depending upon the application.

The GFR α 4s of the present invention can be used to prepare anti-GFR α 4 antibodies. Accordingly, the invention also provides antigenic peptides of GFR α 4. These should preferably comprise at least 8 amino acid residues of the murine or human amino acid sequences defined above and in the claims. These should encompass at least one epitope of GFR α 4 against which an antibody may be raised.

The peptide may form a specific immune complex with GFR α 4. Preferably, the antigenic peptide comprises at least 10, more preferably 15, at least 20 or more amino acid residues providing a sufficiently high antigenicity index. Thus the present invention provides antibodies which specially bind GFR α 4 or GFR α 4-like molecules or compounds interacting with said molecules. The antibody is preferably monoclonal, but naturally polyclonal antibodies may be used. The antibody may be coupled to a detectable or recordable substance and it may be incorporated into a pharmaceutical composition comprising

the antibody and a pharmaceutically acceptable carrier.

Furthermore, the invention pertains to methods for modulating the GFR α 4 mediated cell activity or signalling including cell proliferation and/or differentiation. Such methods include contacting the cell with a compound or agent capable of modulating the activity of a GFR α 4 protein or the expression of GFR α 4 nucleic acid sequence in such a way that a cell associated activity is measurably altered in relation to the cell associated activity in the absence of the agent. The cell, e.g. a neuron or endocrine cell should be capable of responding to a neurotrophic factor through a specific signalling pathway involving the GFR α -4 protein, as described in the examples.

The agents, which modulate GFR α 4 protein can be agonists, which stimulate GFR α 4 activity or the expression of the GFR α 4 nucleic acid sequences or antagonists, which inhibit GFR α 4 protein activity or expression of GFR α 4 nucleic acid sequence.

Examples of agents which stimulate GFR α 4 protein activity or GFR α 4 nucleic acid expression include small molecules and nucleic acid sequences encoding GFR α 4 which can be or have been introduced into the cell. Examples of agents which inhibit GFR α 4 activity or expression include small molecules, antisense GFR α 4 nucleic acid sequences, antibodies specifically binding to GFR α 4. The agent is administered to a cell, such as a neuron or an endocrine cell present in the subject.

The present invention also pertains to methods for making diagnoses and/or treating subjects having disorders mediated by abnormal GFR α 4 activity and/or expression. For example, the invention pertains to methods for treating a subject having a disorder characterized by aberrant GFR α 4 protein activity or nucleic acid expression. Examples of such disorders are

neuronal disorders, endocrine system disorders, e.g., multiple endocrine neoplasia type 2 (MEN2) syndrome, disorders associated with abnormal or aberrant cell development, differentiation and function. For example, axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures may aggravate the disease process. Augmenting GFR α 4 type of signaling in such conditions could be useful by preventing neuronal death without producing aberrant sprouting.

The methods of treatment include administering to the subject GFR α 4 or GFR α 4-like molecules or compounds interacting (modulators or mimetics) with said molecules in such amounts that treatment of the subject occurs.

The invention also pertains to methods for detecting genetic mutations in a GFR α 4 gene, thereby determining if a subject with a mutated gene is at risk of having or is predisposed to have a disorder characterized by aberrant or abnormal GFR α 4 nucleic acid expression or GFR α 4 activity, such as an endocrine system disorder, rare types of MEN2-like syndromes without *Ret* mutations or MEN2-like syndromes with additional phenotype, e.g., pituitary tumours. Activity of GFR α 4 signalling pathway in endocrine systems can lead to disorders including tumours associated with cellular development of cells of these organs as shown in the present RET invention. For example, axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures may aggravate the disease process. Augmenting GFR α 4 type of signaling in such conditions could be useful by preventing neuronal death without producing aberrant sprouting.

The methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic mutation characterized by an alteration affecting the integrity of a gene encoding a GFR α 4 protein, or the misexpression of the GFR α 4 gene.

The invention further pertains to methods for detecting the presence of GFR α 4 or fragment thereof, in a biological sample. The methods involve contacting a biological sample, e.g. any (metastatic) tumour sample, with a compound or an agent capable of detecting GFR α 4 protein or GFR α 4 encoding mRNA in such away that the presence or absence of GFR α 4 can be detected in the biological sample. The compound or agent can be, for example, a nucleic acid probe which is or can be labelled and is capable of hybridizing to GFR α 4 encoding mRNA or an antibody which is labelled or can be labelled with a suitable detectable marker and is capable of binding to a GFR α 4 protein. The invention further provides methods for diagnosing the subject with, for example, an endocrine system disorder or neuron outgrowth disorder based on detection of GFR α 4 protein or its mRNA as well as the GFR α 4 mediated signalling.

The method involves for example the contacting of a cell, tissue, or fluid sample (e.g., a tumour sample) from the subject with an agent capable of detecting GFR α 4 protein or its mRNA and thereafter determining the amount of GFR α 4 protein or mRNA expressed in the sample to a control sample and forming a diagnosis based on the amount of GFR α 4 protein or mRNA expressed in the sample or its signalling as compared to the control sample. Kits for detecting GFR α 4, or fragments thereof by immunoassays or hybridization or microarray techniques from a biological sample are thus also within the scope of the invention.

The present invention also relates to methods, especially, screening assays for identifying compounds or identifying disorders characterized by aberrant GFR α 4 nucleic acid expression or protein activity. Activity of GFR α 4 signalling pathway in endocrine systems can lead to disorders including tumours associated with cellular development of cells of these organs as shown in the present RET invention. For example,

axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures may aggravate the disease process. Augmenting GFR α 4 type of signaling in such conditions could be useful by preventing neuronal death without producing aberrant sprouting.

These methods typically include assaying the ability of a compound or agent to modulate the expression of the GFR α 4 gene or the activity of the GFR α 4 mediated signalling and thereby identifying compounds which are useful for treating a disorder characterized by aberrant GFR α 4 nucleic acid expression or protein activity. The method involves contacting a biological sample obtained from a subject having the disorder with the compound or agent, determining the amount of GFR α 4 protein expressed and/or measuring the activity of the GFR α 4 protein in the biological sample, comparing the amount of GFR4 protein expressed in the biological sample and/or the measurable GFR4 biological activity in the cell to that of a control sample. An alteration in the GFR α 4 expression or GFR α 4 mediated signalling in a cell exposed to a compound or agent in comparison to the control is indicative of a modulation of GFR α 4 expression and/or GFR α 4 activity.

The invention also pertains to methods for identifying compounds or agents which interacts with (e.g., binds to) or mimicks a compound binding to or vice versa a GFR α 4 protein. These methods can include the steps of contacting the GFR α 4 protein or fragments thereof or alternatively a cell expressing GFR α 4 with the compound or agent under conditions which allow binding of the compound to the GFR α 4 protein to form a complex. Thereafter, the formation of the complex comprising the GFR α 4 protein and the compound having the ability of specifically binding to the GFR α 4 protein is measured. The formation of the complex is an indication of the presence of a potentially useful interacting compound. Applicable methods are described in the following patents and patent applications EP 639 584, WO 9638553, US 5,571,506,

WO 200018790, WO 9950439, WO 9945930, US 0,630,619 and WO 9906599.

The invention further pertains to methods for identifying a compound or agent which can modulate, stimulate or inhibit the interaction of the GFR α 4 protein with a target molecule, such as persephin or other neurotrophic factors and the tyrosine kinase receptor RET or other components of signalling pathways and their complexes. In these methods, the GFR α 4 protein is contacted, in the presence of the compound or agent, with the target molecule under conditions which allow binding of the target molecule to the GFR α 4 protein to form a complex. An alteration, e.g., an increase or decrease, in complex formation between the GFR α 4 protein and the target molecule as compared to the amount of the complex formed in the absence of the compound or agent is indicative of the ability of the compound or agent to modulate the interaction of the GFR α 4 protein with a target molecule.

The invention is also related to substances capable of specifically recognizing the GFR α 4 polypeptides defined in above and in the claims. Such substances are for example antibodies, including polyclonal, monoclonal antibodies produced by recombinant DNA techniques or hybridoma techniques as well as ligands capable of binding specifically to the mammalian GFR α 4-receptor. Fragments of antibodies and receptors are also useful as specifically binding substances.

The present invention also enables the production of transgenic non-human animal lacking a functional GFR α 4 receptor and which is useful as a test animal obtainable from a genetically modified compatible cell-line (clone) containing a genomic gene coding for a functional GFR α 4 receptor, which genomic gene may be functionally inactivated or conditionally inactivatable. Said transgenic non-human animal is useful as a test animal or model for studying GFR α 4-mediated signalling in

neuronal cells, endocrine cells, especially in thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, nervous system and testis, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia and for diagnosing and/or treating osteoporosis, endocrine tumours, cancer in parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia. Activity of GFR α 4 signalling pathway in endocrine systems can lead to disorders including tumours associated with cellular development of cells of these organs as shown in the present RET invention. For example, axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures may aggravate the disease process. Augmenting GFR α 4 type of signalling in such conditions could be useful by preventing neuronal death without producing aberrant sprouting.

The present invention also provides a method for obtaining a cell-line (clone) useful in the production of the transgenic non-human animal characterized by having the genomic gene encoding a functional GFR α 4 receptor inactivated by introducing into stem cells a nucleic acid sequence, which is capable of integrating to said genomic *Gfra4* gene and by said integration functionally inactivating said genomic gene or making it conditionally inactivatable.

The cell-line (clone) lacking a functional GFR α 4 receptor is obtainable by established gene targeting methods, by:

- (a) providing a nucleic acid sequence defined in the claims, which sequence is capable of being integrated into the genomic gene coding for a functional GFR α 4 receptor;
- (b) preparing a targeting construct by combining the nucleic acid sequence according to step (a), with a selectable nucleic acid sequence acting as a marker which is capable of functionally inactivating and by said integration functionally

inactivating the genomic gene or making it conditionally inactivatable;

(c) introducing the targeting construct or vector of step (b) into stem cells or cell lines (clones) of the selected animal;

(d) selecting a cell line (clone) containing the nucleic acid sequence or part thereof according to step (a) which has been integrated into the genomic gene of said stem cell or cell line (clone) using a probe, which is capable of recognizing the selectable nucleic acid sequence acting as a marker.

A cell line (clone) obtained by the method described above is lacking a functional GFR α 4 receptor and produces consequently altered levels of GFR α 4. The cell lines (clones) of the present invention are useful not only for producing a transgenic non-human animal but also for studying the effect of different compounds, e.g. obtained by combinatorial chemistry, on GFR α 4-mediated signalling in neurons, endocrine cells, especially in thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, nervous system and testis, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia and for diagnosing and/or treating osteoporosis, endocrine tumours, cancer in parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia.

The invention is described in more detail in the following examples which are intended to illustrate the invention not to limit its scope.

Example 1

Sequence analysis

Several putative exon sequences homologous to known Gfra genes

were identified by BLAST analysis of a 47887 bp sequence containing the putative mouse *Gfra4* locus (Gunn et al., 1999) (Genebank accession number AF155960). Database search using these sequences as query detected three mouse expressed sequence tags (ESTs) matching the 3' end of the putative *Gfra4* gene (AA823200 or AI1553571, a spliced EST from mammary gland; AU035938, an unspliced one from the brain; and AI845209 a short one from the spinal cord). Nucleotide and amino acid sequence comparisons (BestFit), alignment (PileUp), and phylogenetic tree (GrowTree), as well as signal sequence and transmembrane hydrophobicity plots (PeptideStructure) were analyzed using the SeqWeb program package (Genetics Computer Group, Inc.).

Example 2

RACE cloning

cDNA clones representing the 5'-region of *Gfra4* transcript were isolated from adult mouse brain, testis and thyroid gland, as well as from E17 embryo, using the 5'-RACE System (Life Technologies) and the GC-RICH PCR kit (Roche) according to the manufacturer's instructions. Total RNA was extracted with Trizol reagent (Life Technologies). The anti-sense *Gfra4* specific primers used in 5' RACE were: 5'-TTC AGC TCA GTG AGC AGT CAT CG-3' (SEQ ID NO:18:) and second nested primer 5'-CAG GTT GTC CAG GTA GTT GG-3' (SEQ ID NO:19:) or 5'-CAT CGT GCC ACG TAC TCA GA-3' (SEQ ID NO:20:). The 3' region of the *Gfra4* was cloned with 3'RACE method using Clontech Marathon Ready cDNA from E15 mouse embryos according to the manufacturer's instructions. The primers used in 3'-RACE were: 5'-TAC AAG CCT TTG ACA GCT TGC AGC-3' (SEQ ID NO:21:) and a nested one 5'-AGA GCT GGA GGC AGA AAC AGT CC-3' (SEQ ID NO:22:).

Human GFRA4 cDNAs were identified from a human thyroid cDNA (Clontech Marathon Ready cDNA) by 3' and 5'RACE method using the GC-rich PCR kit (Roche) according to the manufacturer's instructions. Reverse transcription reactions were performed

using Superscript II (Life Technologies). PCR systems were GC-RICH PCR kit (Roche) for GFRA4 and PSPN, Expand Long Template PCR system (Roche) for GFRA1, GFRA2, GFRA3 and RET and Dynazyme II (Finnzymes) for PPIA (alias cyclophilin). PCR was run 40 cycles for GFRA4, 35 cycles for PSPN, GFRA1 and GFRA2, and 30 cycles for GFRA3, RET and PPIA using annealing temperatures of 55-60°C. Full-length human GFRA4 cDNA was obtained using Expand High Fidelity system PCR enzyme (Roche) together with GC-RICH PCR system buffers (Roche). PCR products were cloned into mammalian expression vector pCR3.1 (Invitrogen).

Example 3

Northern Blot and Reverse Transcription (RT)-PCR

Northern blot analysis of *Gfra4* expression was carried out from total RNA essentially as described (Rossi et al., 1999). Analysis of the splicing pattern of *Gfra4* was performed by RT-PCR using Superscript II (Life Technologies) for RT and the GC-RICH PCR kit (Roche) for PCR. The following primers were used (see Figure 2C): P1, 5'-CCA CCA TGG CCC ACT GCA TGG AGTC-3' (SEQ ID NO:23:); P2, 5'-CCA CCA TGT TGA GAA GAG CA-3' (SEQ ID NO:24:); P3, 5'-GTG TAC AGC AGA CGA GCG GT-3' (SEQ ID NO:25:); P5, 5'-ATA CAA GCC TTT GAC AGC TTG C-3' (SEQ ID NO:26:); and reverse primers P4, 5'-GTT CCT TGT AAA GAG CTT GCG-3' (SEQ ID NO:27:); and P6, 5'-TGG ACA AGA TGC CTA CTG ACG-3' (SEQ ID NO:28:). The amounts of total RNA was run 35-40 cycles using annealing temperatures of 55-58°C. All the PCR products of different length were sequenced to verify their identity.

Example 4

RNAse Protection Analysis

RNAse protection assays were performed as described (Timmusk et al., 1993) using the RPAII kit of Ambion according to manufacturer's instructions. The template for *Gfra4* specific

cRNA probe was a 550 bp PCR fragment generated using primers P3 and P4. It yielded a fully protected fragment corresponding to *Gfra4* mRNA that have spliced both introns between exons II and IV, as well as 235 bp and 315 bp fragment corresponding to *Gfra4* mRNA that is unspliced between exons II and III.

Example 5

***In Situ* Hybridization**

In situ hybridizations for *Ret*, *Gfra1* and *Gfra2* was performed as described Rossi et al., 1999). A mouse full-length *Gfra3* cDNA (AF051766) and a bp cDNA fragment of the 3'-end of the mouse *Gfra4* was used as template for sense and anti-sense RNA probes. Control sections hybridized with sense probe did not show labeling above background (not shown).

In situ hybridizations for RET, GFRA1, GFRA2, GFRA3 and GFRA4 were performed principally in the same way as described above on cryosections of the same thyroid tumor samples analyzed by RT-PCR. A 206 bp cDNA fragment of the 5'-end of human GFRA4 was used as template for sense and antisense RNA probes. PCR fragments of GFRA1, GFRA2, GFRA3 and RET generated with the same primers as in RT-PCR were cloned and sequenced and used as templates for antisense and sense RNA probes. Control sections hybridized with sense probe did not show labeling above background (not shown). Dark-field and corresponding bright-field images of Nissl counter-stained sections were digitized and processed using Adobe PhotoShop software.

Example 6

Immunohistochemistry

Ret-deficient (-/-) and control littermate mice were genotyped as described (Durbec et al., 1996). Anesthetized day E18 embryos were perfused transcardially with 4% paraformaldehyde in PBS, postfixed for 2 hours and cryoprotected overnight in 25% sucrose. Horizontal cryosections of the thyroid gland,

serially sectioned at 15 μ m, were stained and analyzed by standard immunofluorescence techniques. DAPI (Sigma) was included in embedding medium to stain the cell nuclei. The primary goat polyclonal antibody against calcitonin (sc-7784) was from Santa Cruz Biotechnology. All immunopositive profiles with a clear nucleus were counted in every section through the thyroid glands. The mean nuclear diameter of the C-cells was similar (<1% difference) between the Ret -/- and control groups (not shown). Raw profile numbers per animal without correction are given as mean \pm S.D.

Example 7

RNA isolation

RNA from different human tissues was purchased from Clontech (Human Total RNA Panel I-V). Total RNA from frozen human thyroid tissue was isolated using UltraspecTM-II RNA isolation system (Biotecx Laboratories, Inc.). Total RNA from the TT cell-line (ATCC#CRL1803) was isolated with Trizol reagent (Life Technologies). The RET mutation of five MTC patients had been analyzed: one from a MEN2A family member contained the germline C634R mutation, one patient was positive for the M918T but the germline situation was unknown, and three were sporadic MTCs of which one was positive for the M918T mutation.

Example 8

RT-PCR

The antisense primers used in 5'RACE were mouse specific Gfra4 primer 5'-CAC GTT GTC CAG GTA GTT GG-3' (SEQ ID NO:41:) and a second human specific GFRA4 nested primer 5'-GCA CTG CGC CAC ATA CTC GGA-3' (SEQ ID NO:42:). The 3'region of GFRA4 was cloned in two steps, first using a GFRA4 specific sense primer P2 (see Figure 1A) 5'-GCT CCG AGT ATG TGG CGC AGT-3' (SEQ ID NO:43:) and a nested primer 5'-GCT CAC CCA CGC ACT GCT CTT CTG-3' (SEQ ID NO:44:). The 3'region containing the stop codon

and 3'UTR sequence was cloned using a sense primer 5'-CCT AAC TAC GTG GAC AAC GTG AGC-3' (SEQ ID NO:45:) and a second nested primer 5'-ATG GTG CCA TTC AGG CCT TTG CCA G-3' (SEQ ID NO:46:) or 5'-GCA GGT GTC CTC CAC AGG CAG-3' (SEQ ID NO:47:). RT-PCR of genomic human DNA was used to locate the intronic nucleotide sequences between exons 4 and 5, and between exons 5 and 6, using a sense primer 5'-CCT AAC TAC GTG GAC AAC GTG AGC-3' (SEQ ID NO:45:) and an antisense primer 5'-GAA GTA TGG AGA GCA GGG AGC GTC-3' (SEQ ID NO:48:). Full-length human GFRA4 cDNAs were obtained using a sense primer P1 (see Figure 9A); 5'-CCA CCA TGG TCC GCT GCC TGG-3' (SEQ ID NO:49:) and an antisense primer P3; 5'-GAG GTC GCT GTC CTA ATC AGA G-3' (SEQ ID NO:50:).

Primers used in RT-PCR for human, mouse and rat PSPN were as described in (Milbrandt, et al., 1998). Primers used in RT-PCR for human GFRA1 flanked nucleotides 491-870 of GenBank sequence AF042080, GFRA2 flanked nucleotides 148-427 of GenBank sequence U93703, GFRA3 flanked nucleotides 574-1203 of GenBank sequence NM001496 and RET flanked nucleotides 833-1114 of GenBank sequence X12949. The amounts of total RNA in samples were normalized by amplification of a PPIA fragment.

Example 9

¹²⁵I-labelled PSPN Binding

Murine neuroblastoma Neuro-2a cells were transfected (FuGene6, Roche) with full-length GFRA4a cDNA in pCR3.1 vector (Invitrogen), and bulk-selected with 400 (g/ml of G418. PSPN was enzymatically iodinated by lactoperoxidase, up to a specific activity of 100.000 cpm/ng. Binding assays were performed essentially as described (Laurikainen, et al., 2000) with 0.9 nM [¹²⁵I] PSPN in binding buffer (DMEM containing 0.2% BSA and 15 mM Hepes, pH 7.5) for 4 hrs on ice, either in the presence or absence of different concentrations of unlabeled human PSPN (PeproTech EC Ltd.), rat GDNF (Cephalon, Inc.), human NRTN (PeproTech EC Ltd.) or human ARTN (a gift

from Drs. J. Milbrandt and E.M. Johnson, Jr.). The amount of [^{125}I]PSPN bound to mock-transfected cells was at the background level. KD was calculated using Cheng-Prusoff equation (McGonigle, et al., 1994).

Example 10

Chemical cross-linking and immunoprecipitation

CHO cells were transfected (FuGene 6) with full-length human GFRA4a cDNA, grown for two days, washed and incubated with 0.9 nM [^{125}I]PSPN in binding buffer (see above) on ice for 4 hrs. After washing the cells were incubated with 1 mM Bis suberate (Pierce) in PBS at RT for 25 min. Following washes, some samples were treated with 0.5 U/ml of PI-PLC (Sigma) at +37 °C for 30 min. Cells were lysed in Laemmli buffer containing β -mercaptoethanol.

The cross-linking reaction for human GFRA4a and mock transfected (pcDNA3; Invitrogen) Neuro-2a cells was done as described above but the cells were lysed in NP-40 lysis buffer (1X Tris-borate saline, 2 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, 1 mM PMSF, CompleteTM protease inhibitor cocktail, Roche), clarified by centrifugation at 14 000 rpm for 20 min., immunoprecipitated with 1 (g/ml of Ret antibodies (Santa Cruz Biotechnology) and collected with Protein A Sepharose. The immunocomplexes were washed with NP-40 buffer, and separated by 7.5% SDS-PAGE.

Example 11

Ret Phosphorylation Assay

Semiconfluent human GFRA4a-, rat Gfra1- or vector-expressing Neuro-2a cells were maintained with 0.5% serum for 24 hr and then in serum free medium for 4 hr prior to stimulation. After stimulation with PSPN (0.01-100 ng/ml) or GDNF (100 ng/ml), cells were lysed and precipitated with agarose-conjugated anti-phosphotyrosine antibodies (4G10, Upstate Biotechnology)

overnight at +4 °C on ice. The membranes were probed with 60 ng/ml anti-Ret antibodies (Santa Cruz).

Example 12

Neuronal Microinjections

Postnatal day 1-2 superior cervical ganglion (SCG) neurons were grown 5-6 days on polyornithine-laminin-coated dishes with NGF (30 ng/ml). 50:50 ng/ μ l cDNA mixtures of short isoform of human RET (gift from Dr. M. Billaud) and either mouse Gfra4a1 (in pcDNA3, Invitrogen) or human GFRA4a (in pCR3.1, not shown) or empty pcDNA3 vector, were pressure-microinjected into nuclei. To find successfully injected neurons later, a plasmid encoding green fluorescent protein (10 ng/ μ l) was included in every injection mixture. Neurons were grown overnight with NGF and thereafter in NGF-free medium containing blocking anti-NGF antibodies (Boehringer-Mannheim) and PSPN, GDNF, NRTN or ARTN, all at 100 ng/ml. Number of living fluorescent neurons or uninjected control neurons was then counted (initial neurons). 30-80 initial neurons were successfully injected for every treatment group. Healthy fluorescent neurons with intact nuclei and phase-bright cytoplasm were counted 70-75 h later and expressed as percent of initial neurons. Experiments were repeated on independent cultures: n=7 for PSPN, n=4 for GDNF and NRTN, n=3 for ARTN. Significance of the differences between means was estimated by one-way ANOVA followed by Tuckey's post-hoc test at the significance level of $\alpha=0.05$.

Example 13

Analyzing changes in phosphorylation status

To analyze changes in the phosphorylation status of MAPK (ERK1 and ERK2) and Akt (PKB) in Neuro-2a cell lines stable transfected with either hGFR α 4, GFR α 1 or empty vector, the semiconfluent cells were kept for 18 hrs in the culture medium containing 0.5% fetal calf serum and then were starved for 3

hours in serum-free medium. After stimulation with GDNF or PSPN, the cells were briefly washed with PBS containing sodium vanadate and sodium fluoride and lysed in NP-40 lysis buffer (Tris-buffered saline; 2 mM EDTA; 1% NP-40; 1% Triton X-100; 1 mM PMSF; 1 mM Na_3VO_4 ; 25 mM NaF; Complete™ protease inhibitors cocktail (Boehringer Mannheim). The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL (Amersham) membrane. Immunoblotting was carried out using either MAPK (ERK1/2) Anti-Active™ (Promega) or anti-Akt antibodies (New England Biolabs) recognizing phosphorylated forms of the kinases, and detected with ECL system (Amersham). The blots were then reprobed with MAPK or Akt phosphorylation-independent antibodies.

Example 14

Neurite outgrowth assay

To assay neurite outgrowth, cells were plated at 0.5×10^5 cells/cm² and maintained in RPMI-1640 containing 1 fetal calf serum supplemented with or without 100 ng/ml either GDNF or PSPN. After 72 hours the percentage of cells with neurites (i.e. bearing processes longer than cell body diameter) was determined. To assay cell survival, cells were washed with serum-free medium, detached and plated in 24-well tissue culture dishes (at approximately 0.5×10^5 cells/cm²) containing serum-free medium supplemented with either GDNF or PSPN at different concentrations. Cell viability was determined at day 3 after plating, using Trypan Blue dye exclusion method.

The results of the experiments carried out during the studies are disclosed in more detail below.

The Description of the Drawings

Figure 1. Sequence of the predicted GPI-linked mouse GFR α 4 protein (SEQ ID NO:2:) and structure of mouse *Gfra4* gene.

Figure 1A. Alignment of mouse GFR α 1, GFR α 2, GFR α 3, and GFR α 4 and chicken GFR α 4 amino-acid sequences. Predicted signal sequences are underlined. Cysteines are highlighted. The sequence was divided between the predicted hinge regions into four parts and the two cysteins in the homologous Cys-rich domains (D1-D3, see Figure 1D) were aligned manually. The last two amino-acids coded by exons II and exon V are marked by asterisk.

Figure 1B. Phylogenetic tree of the GFR α family. Comparisons were made by using the protein encoding region of mouse *Gfra1-Gfra4* cDNAs.

Figure 1C. Structure of mouse *Gfra4* gene (upper) as compared to mouse *Gfra2* gene (lower) that has alternatively spliced exons 2 and 3 (Baloh et al., 1998). The structures of mouse *Gfra3* (Baloh et al., 1998) and human GFRA1 genes (Angrist et al., 1998) are similar to the mouse *Gfra2* gene, except for an additional short exon that encodes for hinge region in GFRA1 and lack of exon 8 in *Gfra3*. The intron separating exons II and III is very short (53 bp) in *Gfra4* as compared to this intron in other *Gfra* genes (>2 kb). Exon grey shades refer to the homologous Cys-rich domains (D1-D3) and hinge regions in the domain model. N- and C-terminal signal sequences are lined. The lengths of the 5' UTR sequences of exons Ia and Ib (broken line) were not determined.

Figure 1D. Proposed domain structure of mouse GFR α 4 (left) and GFR α 2 receptor (right) (Airaksinen et al., 1999). Mouse GFR α 4 lacks the first domain (D1), which is present in other GFR α -receptors. Putative N-glycosylation sites are also indicated. (>-)

Figure 2. Expressions and developmentally regulated tissue-specific splicing of *Gfra4* gene.

Figure 2A. Northern blot analysis of *Gfra4* expression in various mouse tissues (upper). The major transcript in testis appears smaller (appr. 1.4 kb). Longer transcripts (appr. 1.6 kb and 2.5 kb) are also seen. Rehybridization with GAPDH probe (below) demonstrates equal loading.

Figure 2B. Semiquantitative RT-PCR analysis of *Gfra4* transcripts from various tissues and postnatal stages. Locations of PCR primers are indicated on the right and shown in Figure 2C. *Upper panel:* Transcripts containing exon Ia and the spliced intron between exons II and III (Ia/ Δ i 703 bp) are produced in adrenal, pituitary and thyroid glands but not in brain or testis. *2nd upper panel:* mRNAs containing exon Ib (Ib/ Δ i 676 bp) show a expression pattern similar to those containing exon Ia. *Middle panel:* The short intron between exons II and III is efficiently spliced (Δ i 550 bp) in thyroid and pituitary glands but not (i 603bp) in brain and testis. *2nd lower panel:* 3-week-old thyroid glands express the shorter (GPI, 315 bp) splice form, whereas newborn and 6-week-old thyroid glands, preferably as well as postnatal adrenal and pituitary glands, produce the longer (TM 380 bp) splice form. Note that the testis and brain also express their TM or GPI splice forms, but protein synthesis would produce a putative soluble form due to intron inclusion. *Lower panel:* The lines contain similar levels of control *Ppian* transcript.

Figure 2C. Structures of different *Gfra4* mRNA splice forms containing exon Ia (a1-a4) and exon Ib (b1-b4). Primers P1-P6 used in Figure 2B are indicated below the gene structure. Asterisks denote stop codons. The exon colours are the same as in Figures 1C and 1D. Transcript a1 (SEQ ID NO:7:) is predicted to encode for the GPI-linked isoform of GFR α 4 (SEQ ID NO:7:), whereas a2 (SEQ ID NO:8:) encodes for a putative transmembrane (TM)-anchored receptor (SEQ ID NO:2:). Transcripts a3 and a4 (SEQ ID NO:9:) encode for truncated and secreted molecule (domain-2 only) (SEQ ID NO:3:). Transcripts b1-b4 are similar to the transcripts a1-a4 except for usage of exon Ib,

which encodes for a putative weak N-terminal signal sequence instead of exon 1a. Shown in Figures 2A and 2B are representative results from one of three independent experiments. Abbreviations: ad, adult; pit, pituitary gland.

Figure 3. Intron-exon junctions in the coding region of mouse *Gfra4* gene. Consensus splicing sequences are indicated (upper line). There are more non-canonical nucleotides (marked in bold) in the splicing signals of the alternatively spliced introns. Splice acceptor and donor sites are shaded.

Figure 4. Localization of *Gfra4* and *Ret* mRNAs in developing and mature mouse tissues by *in situ* hybridization.

Figure 4A. *Gfra4* is highly expressed in the intermediate lobe (Im1) of E18 pituitary gland (sagittal section), while moderate *Ret* expression is seen in the neurohypophysis (Nh). Ah, adenohypophysis.

Figure 4B. *Gfra4* is highly expressed in the intermediate lobe (Im1) of E18 pituitary gland (sagittal section), while moderate *Ret* expression is seen in the neurohypophysis (Nh). Ah, adenohypophysis.

Figure 4C. The condensing mesenchyme of developing limbs (arrows) shows moderate *Gfra4* expression at E16.

Figure 4D. Many neurons of adult spinal cord (coronal section) show moderate to high *Gfra4* expression.

Figure 4E. In adult brain (horizontal section), the distribution of *Gfra4* and *Ret* mRNAs are not overlapping. *Gfra4* is highest in the hippocampus (Hc). Cb, cerebellum.

Figure 4F. In adult brain (horizontal section), the distribution of *Gfra4* and *Ret* mRNAs are not overlapping. *Gfra4* is highest in the hippocampus (Hc). Cb, cerebellum.

Figure 4G. The circular muscle layer and the myenteric ganglia of the adult colon express *Gfra4*. H & E staining.

Figure 4H. The circular muscle layer and the myenteric ganglia of the adult colon express *Gfra4*. Dark field image.

Figure 4I. Seminiferous tubules in adult testis express *Gfra4*. H & E staining.

Figure 4J. Seminiferous tubules in adult testis express *Gfra4*. Dark field image.

Figure 5. *Gfra4* and *Ret* are co-expressed in mouse thyroid C-cells and adrenal chromaffin cells.

Figure 5A. *In situ* hybridization of adjacent sagittal sections of E12 mouse embryos for *Gfra4* (Figure 5A) and *Ret* (Figure 5C). Arrowheads point to the ultimobranchial body. drg dorsal root ganglia.

Figure 5B. *In situ* hybridization of adjacent sagittal sections of E12 mouse embryos for *Gfra4* (Figure 5A) and *Ret* (Figure 5C). Arrowheads point to the ultimobranchial body. drg dorsal root ganglia.

Figure 5C. *In situ* hybridization of adjacent sagittal sections of E12 mouse embryos for *Gfra4* (Figure 5A) and *Ret* (Figure 5C). Arrowheads point to the ultimobranchial body. drg dorsal root ganglia.

Figure 5D. Adjacent sagittal sections of E18 embryos hybridized for *Gfra4* (Figure 5D) and *Ret* (Figure 5F). Both *Gfra4* and *Ret* are expressed in the C-cells but not in the parathyroid gland (broken line).

Figure 5E. Adjacent sagittal sections of E18 embryos

hybridized for *Gfra4* (Figure 5D) and *Ret* (Figure 5F). Both *Gfra4* and *Ret* are expressed in the C-cells but not in the parathyroid gland (broken line).

Figure 5F. Adjacent sagittal sections of E18 embryos hybridized for *Gfra4* (Figure 5D) and *Ret* (Figure 5F). Both *Gfra4* and *Ret* are expressed in the C-cells but not in the parathyroid gland (broken line).

Figure 5G. In 6-week-old mice, *Gfra4* is highly expressed in the thyroid parafollicular C-cells (arrow), but also in the parathyroid gland (broken line). In contrast, *Ret* expression is detectable only in the C-cells (arrow) but not in the parathyroid gland.

Figure 5H. In 6-week-old mice, *Gfra4* is highly expressed in the thyroid parafollicular C-cells (arrow), but also in the parathyroid gland (broken line). In contrast, *Ret* expression is detectable only in the C-cells (arrow) but not in the parathyroid gland.

Figure 5I. In 6-week-old mice, *Gfra4* is highly expressed in the thyroid parafollicular C-cells (arrow), but also in the parathyroid gland (broken line). In contrast, *Ret* expression is detectable only in the C-cells (arrow) but not in the parathyroid gland.

Figure 5J. *Gfra4* is highly expressed by all cells in adrenal medulla at postnatal day P8, while only a subpopulation of them express *Ret*.

Figure 5K. *Gfra4* is highly expressed by all cells in adrenal medulla at postnatal day P8, while only a subpopulation of them express *Ret*.

Figure 5L. *Gfra4* is highly expressed by all cells in adrenal medulla at postnatal day P8, while only a subpopulation of

them express *Ret*. The bright-field images shown in Figures 5B, 5E, 5H, and 5K correspond to those in 5A, 5F, 5I and 5L, respectively.

Figure 6. *Ret*-deficient mice have less thyroid calcitonin-cells.

Figure 6A. E18 thyroid glands from wild-type (Figure 6A) and *Ret*-deficient (Figure 6B) mice are immunostained for calcitonin. Shown are representative sections containing the highest number of labeled C-cells through the middle part of the thyroid gland. The remaining C-cells in *Ret*-deficient mice appear morphologically normal.

Figure 6B. E18 thyroid glands from wild-type (Figure 6A) and *Ret*-deficient (Figure 6B) mice are immunostained for calcitonin. Shown are representative sections containing the highest number of labeled C-cells through the middle part of the thyroid gland. The remaining C-cells in *Ret*-deficient mice appear morphologically normal.

Figure 7. RT-PCR analysis of *GFRA4* expression in human thyroid tumors. Each line represents analysis of a tumor from a different patient. The specific 264 bp PCR product representing *GFRA4* (exon 1a) expression is high in all medullary carcinoma samples (3/3) but not detectable in other thyroid tumors tested including, 4 follicular adenoma, 4 follicular carcinoma and 5 papillary carcinoma samples.

Figure 8. Localization of human *GFRA4* mRNA expression in medullary thyroid carcinoma but not in other thyroid tumors. *In situ* hybridization was performed using a human *GFRA4* specific antisense cRNA probe on frozen sections.

Figure 8A. *GFRA4* is highly expressed by virtually all cells in the medullary thyroid carcinoma (MTC) but not in the surrounding connective tissue (dark field image).

Figure 8B. Light-field image shows the Nissl-stained structure of tissue.

Figure 8C. Other thyroid tumors, including follicular adenoma (FTA) (Figures 8C and 8D) and papillary carcinoma (PTC) (Figures 8E and 8F) show no *GFRA4* expression above sense control levels (not shown).

Figure 8D. Other thyroid tumors, including follicular adenoma (FTA) (Figures 8C and 8D) and papillary carcinoma (PTC) (Figures 8E and 8F) show no *GFRA4* expression above sense control levels (not shown).

Figure 8E. Other thyroid tumors, including follicular adenoma (FTA) (Figures 8C and 8D) and papillary carcinoma (PTC) (Figures 8E and 8F) show no *GFRA4* expression above sense control levels (not shown).

Figure 8F. Other thyroid tumors, including follicular adenoma (FTA) (Figure 8C and 8D) and papillary carcinoma (PTC) (Figures 8E and 8F) show no *GFRA4* expression above sense control levels (not shown).

Figure 9. Structure of the human *GFRA4* gene compared to mouse *Gfra4* and different splice forms of human *GFRA4* mRNA.

Figure 9A. Structure of the human *GFRA4* gene (top) compared to mouse *Gfra4* gene (bottom). Both contain at least 6 exons but no alternative exon 1b has been found in human. Also the putative transmembrane form encoded by the alternatively spliced exon 6 found in mouse is absent in human. Primers P1-P3, used in RT-PCR in Figures 2 and 5 are marked by arrowheads.

Figure 9B. Splicing of *GFRA4* gene in human thyroid gland. Two GPI-linked isoforms (*GFRA4a* and *GFRA4b*), and one putative

soluble isoform (*GFRA4c*) are produced by alternative splicing. In transcript encoding for *GFR α 4a* all the introns are spliced, whereas *GFR α 4b* contains the intron between exon 2 and exon 3. Transcript encoding *GFR α 4c* contain introns between exons 2 and 3 and between exons 3 and 4. Asterisk marks the stop codon.

Figure 9C. Proposed schematic domain structure of human *GFR α 4* isoforms.

Figure 10. RT-PCR analysis of *GFRA4*, *PSPN* and *RET* transcripts in various human tissues. Top row: *GFRA4* expression is detected only in thyroid gland using primers P2 and P3 (see Figure 15A). All the introns in the transcript corresponding to 699 bp are spliced. The 789 bp transcript contains a small intron between exon 2 and exon 3 and corresponds to the *GFR α 4b* form. The 863 bp fragment contains introns between exons 2 and 3 and between exons 3 and 4, and corresponds to the soluble *GFR α 4c* form. Second row: An unspliced transcript of the human *PSPN* is expressed in all tissues (approximately 590 bp, intron size not determined), whereas the spliced transcript (476 bp), which corresponds to the functional *PSPN*, is weakly expressed in cerebellum, adrenal gland and testis and barely detectable in spinal cord. To exclude DNA contamination in RT-PCR reactions, controls containing RNA were also subjected to PCR (not shown). Third row: *RET* expression (282 bp) is detectable in almost all human tissues examined but the expression levels are strongest in adult brain, cerebellum, spinal cord, salivary gland, adrenal gland and prostate. Bottom row: Control RT-PCR with *PPIA* transcript showing equal loading of cDNA. In all PCR experiments, negative water controls were included (not shown).

Figure 11. Binding and Chemical Cross-linking of [¹²⁵I]*PSPN* to Human *GFR α 4* and Ret.

Figure 11A. Displacement binding of [¹²⁵I]*PSPN* by *PSPN*, *NRTN*, *GDNF* and *ARTN* from human *GFR α 4a*-expressing Neuro-2a cells,

shown as percentage of [^{125}I]PSPN bound in the absence of a cold ligand (100%). One representative experiment out of four is shown.

Figure 11B. Chemical cross-linking of [^{125}I]PSPN to vector- (lanes 1, 2) or hGFRA4a transfected (lanes 3-6) CHO cells, in the presence (lanes 2, 3, 5) or absence (lanes 1, 4, 6) of unlabeled PSPN, treated (lanes 3, 4) or not treated (lanes 1, 2, 5, 6) with PI-PLC. Bands are designated as follows: arrowhead, approximately 46 kD; arrow, approximately 62 kD; asterisk, approximately 92 kD.

Figure 11C. Proteins cross-linked to [^{125}I]PSPN in the presence (lanes 1, 3) or absence (lanes 2, 4) of unlabeled PSPN in vector- (lanes 1, 2) or GFRA4a- (lanes 3, 4) transfected Neuro-2a cells. Bands are designated as in Figure 11B.

Figure 11D. Immunoprecipitation of Ret from vector- (lanes 1, 3) or GFRA4a-transfected (lanes 2, 4) Neuro-2a cells after chemical cross-linking of [^{125}I]PSPN, separated in the presence (lanes 1, 2) or absence (lanes 3, 4) of (β -mercaptoethanol (β -Me). Bands are designated as follows: arrowhead, approximately 46 kD; arrow, approximately 196 kD; diamond approximately 232 kD; asterisk, approximately 400 kD.

Figure 11E. Phosphorylation of Ret in mock- (lanes 1, 2), GFRA4a- (lanes 3, 4, 5) or Gfra1-transfected Neuro-2a cells upon stimulation with 100 ng/ml of PSPN (lanes 2, 4, 5) or 100 ng/ml of GDNF (lane 7).

Figure 11F. Dose-dependent phosphorylation of Ret with PSPN in hGFRA4a-transfected Neuro-2a cells.

Figure 12. PSPN specifically promotes survival of RET/Gfra4-injected SCG neurons. Neonatal mouse SCG neurons

were grown five days with NGF, injected with indicated plasmid mixtures (all 50:50 ng/ μ l), grown overnight with NGF, then NGF was changed for indicated factors (all 100 ng/ml). Numbers of viable neurons after 3-day treatment period are expressed relative to the number of initial neurons. Means \pm s.e.m of three to seven independent repeats for each factor is shown. $p < 0.05$ (*); $p < 0.001$ (***) .

Figure 13. Semiquantitative RT-PCR analysis of *GFRA4*, *PSPN* and *RET* in different human thyroid tumors. Top row: *GFRA4* mRNA is selectively expressed in medullary thyroid carcinomas (MTC). No *GFRA4* transcripts are detectable in follicular thyroid adenomas (FTA), follicular thyroid carcinomas (FTC) or papillary thyroid carcinomas (PTC). Each lane represents tumor cDNA from different patient. Normal thyroid gland, including medullary tissue (NT) also expresses *GFRA4*. Two major transcripts of 831 and 910 bp seen in MTC and TT-cells correspond to *GFR α 4a* and *GFR α 4b*, respectively (primers P1 and P3, see Figure 15A). The minor transcript of 999 bp corresponds to the soluble *GFR α 4c*. Second row: No spliced form of the *PSPN* transcript is detectable using primers covering the initiation and the stop codons. Third row: *RET* mRNA is expressed in all MTC samples. Bottom row: Control RT-PCR with *PPIA* transcript showing equal loading of cDNA. All experiments contained negative water controls (not shown).

Figure 14. Localization of *GFRA1*, *GFRA2*, *GFRA3*, *GFRA4* and *RET* mRNAs in thyroid tumors by *in situ* hybridization.

Figure 14A. Typical representative examples of autoradiography films showing *GFRA1*-*GFRA4* and *RET* cRNA hybridization to adjacent frozen sections of different thyroid tumors. Of the four types of tumors represented, only the MTC sections were strongly stained for *GFRA4* and *RET* expression. *GFRA1*, *GFRA2*, *GFRA3* mRNA levels were very low or undetectable in all samples including MTC.

Figure 14B. *GFRA4* and *RET* probes were hybridized to frozen sections representing follicular thyroid adenoma (FTA), follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), and medullary thyroid carcinoma (MTC) samples and sections were counter-stained by Nissl-substance. *GFRA4* and *RET* is highly expressed in all malignant cells of the MTC sample but not in the surrounding connective tissue. Bar in A= 0.5 cm, in B=100 μ m.

Figure 15. Activation of Akt in Neuro-2a cells expressing either GFR α 1 or hGFR α 4 with their respective ligands.

Figure 15A. Neither GDNF nor PSPN (native or heat-inactivated) stimulate Akt phosphorylation in mock-transfected Neuro-2a cells. In Neuro-2a cells expressing GFR α 1, GDNF, but not PSPN evokes Akt phosphorylation.

Figure 15B. PSPN, but not GDNF, stimulates Akt phosphorylation in Neuro-2a cells expressing GFR α 4. Upper panels in Figure 15A and Figure 15B show immunoblotting of the cell lysates with anti phospho-Akt (Ser 473) antibodies. Lower panels show the re-probing of the same blots with phosphorylation state-independent anti Akt antibodies.

Figure 16. MAP kinases are activated in response to GDNF but not to PSPN in the Neuro-2a cells.

Figure 16A. Neither GDNF, nor PSPN (native or heat-inactivated), stimulate MAPK phosphorylation in mock-transfected Neuro-2a cells. In Neuro-2a/GFR α 1 cells GDNF, but not PSPN, activates MAPK.

Figure 16B. In Neuro-2a/hGFR α 4 cells PSPN is unable to stimulate phosphorylation of MAPK. Upper panels in Figure 16A and Figure 16B show immunoblotting of the cell lysates with anti phospho-MAPK antibodies. Lower panels show the re-probing

of the same blots with phosphorylation state-independent anti MAPK antibodies.

Figure 17A. GDNF and PSPN both stimulate cell survival in dose-dependent manner via their respective receptors in serum-free conditions.

Figure 17B. PSPN, in contrast to GDNF, cannot induce neurite outgrowth in Neuro-2a/hGFR α 4 cells. Shown are mean values with SEM. ** - $P < 0.01$; *** - $P < 0.001$.

Figure 18. cDNA and amino-acid sequences of mouse GFR α 4 GPI-isoform a1. The GPI-linked isoform, (cf. Figure 1A) corresponds to transcript a1 in Figure 2C. The signal sequence encoded by exon 1a is underlined.

Figure 19. The putative TM-anchored isoform, GFR α 4-a2, encoded by transcript a2 in Figure 2C.

Figure 20. cDNA and amino-acid sequence of GFR α 4-a3/4, a soluble isoform of mouse GFR α 4. The signal sequence encoded by exon 1a is underlined. GFR α 4-a3/4 is encoded by transcripts a3-a4 as shown in Figure 2C.

Figure 21. Shown are the cDNA and amino-acid sequence of human GFR α 4 a GPI-linked isoform a.

Figure 22. Human GFR α 4 b, putative GPI-linked isoform b.

Figure 23. Human GFR α 4 c, a putative secreted isoform, formed by alternative splicing of the second short intron.

Figure 24. Genomic sequence of human GFRA4 locus. The coding sequences are in uppercase. Intronic sequences are in lowercase and bold. The alternatively spliced 3'-end of the 2nd short intron is shown in italics.

Table 1

Expression of *Gfra4* and *Ret* mRNAs in Selected Mouse Tissues and Cells

Age	E12		E16		P0		P20		adult	
Area	<i>Gfra4</i>	<i>Ret</i>	<i>Gfra4</i>	<i>Ret</i>	<i>Gfra4</i>	<i>Ret</i>	<i>Gfra4</i>	<i>Ret</i>	<i>Gfra4</i>	<i>Ret</i>
NC	±	-	++	-	++	-	nd	nd	++	-
HC	nd	nd	nd	nd	++	-	nd	nd	+++	+
VM	+	+++	nd	nd	+	+++	nd	nd	+	+++
VSC	+	+++	++	+++	+	+++	nd	nd	+	+++
PNS	±	+++	+	+++	++	+++	++	+++	++	+++
PI	nd	nd	+++	-	+++	-	+++	nd	+++	-
TC	++	++	+++	++	+++	++	+++	++	+++	+
PT	nd	nd	-	-	±	-	+++	-	+++	-
AdM	nd	nd	nd	nd	++	++*	++	+	++	+
Kidney	+	+++	+	+++	±	+++	-	+	-	±
Lung	+	+	+	+	±	-	nd	nd	-	-
Liver	-	-	-	-	-	-	nd	nd	-	-
Testis	nd	nd	nd	nd	+	++*	++	±	++	±

mRNA expression was graded from -, no labeling above background, to + + +, highest detected expression level based on visual inspection of *in situ* hybridization sections. Expression of *Gfra4* and *Ret* at E14 and E16 as well as P8 and P20 were overlapping.

*Expression differs from that reported by Golden et al., 1999.

Abbreviations

IML, intermediate lobe; nd, not determined; PNS, peripheral nervous system including sensory (DRG), sympathetic (SGG) and enteric ganglia; NC, Neocortex; HC, Hippocampus; VM, Ventral Midbrain; VSC, Ventral Spinal Cord; PNS, PNS ganglia; PI, Pituitary (IML); TC, Thyroid (C-cells); PT, Parathyroid; AdM, Adrenal Medulla; nd, not determined.

Table 2

Expression of human *GFRA1*, *GFRA2*, *GFRA3*, *GFRA4* and *RET* in thyroid tumours and normal thyroid

Tumour	<i>GFRA1</i>		<i>GFRA2</i>		<i>GFRA3</i>		<i>GFRA4</i>		<i>RET</i>	
	RT	<i>In situ</i>	RT	<i>In Situ</i>	RT	<i>In situ</i>	RT	<i>In situ</i>	RT	<i>In situ</i>
FTA	+	+	+	+	+	+	-	-	-	-
	1/4	1/2	3/4	2/2	4/4	2/2	0/4	0/3	0/4	0/2
FTC	+	-	+	+	+	+	-	-	-	-
	1/4	0/1	2/4	1/1	2/4	1/2	0/4	0/3	0/4	0/1
PTC	+	-	+	+	+	+	-	-	-	-
	2/5	0/2	3/5	2/2	2/5	1/3	0/5	0/4	0/5	0/2
MTC	+	+	+	+	+	+	+++	+++	+++	+++
	5/8	4/6	7/8	4/6	4/8	2/6	8/8	6/6	8/8	6/6
NT	+	Nd	+	Nd	+	Nd	++	Nd	++	Nd
	1/1		1/1		2/3		1/3		1/1	
TT cells	+	Nd	-	Nd	-	Nd	+++	Nd	+++	Nd

mRNA expression is graded from -, no labelling above background, to +++, highest detected expression based on visual inspection. The numbers below the tissue gradings denote the number of samples positive/total number of samples analyzed.

Abbreviations

RT, RT-PCR; FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; MTC, medullary thyroid carcinoma; NT, normal thyroid; Nd, not determined.

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What is claimed:

1. An isolated and purified nucleic acid sequence, characterized in that the nucleic acid sequence is a cDNA encoding a splicing isoform of mammalian GFR α 4 comprising the amino acid sequence (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) or fragments thereof.
2. The nucleic acid sequence according to claim 1, characterized in that the nucleic acid sequence is a cDNA encoding a splicing isoform of murine GFR α 4 comprising the amino acid sequences (SEQ ID NO:1:), (SEQ ID NO:2:) or (SEQ ID NO:3:) or fragments thereof.
3. The nucleic acid sequence according to claim 1, characterized in that the nucleic acid sequence is a cDNA encoding a splicing isoform of human GFR α 4 comprising of the amino acid sequence (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:) or fragments thereof.
4. The nucleic acid sequence according to claim 1, characterized in that the nucleic acid sequence is a cDNA having the sequence (SEQ ID NO:7:), (SEQ ID NO:8:), (SEQ ID NO:9:), (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:) or fragments thereof, encoding a polypeptide having substantially similar functions as human or mouse GFR α 4.
5. The nucleic acid sequence according to claim 1, characterized in that the nucleic acid sequence is a cDNA encoding a human GFR α 4 characterized by comprising the nucleic acid sequence (SEQ ID NO:10:), (SEQ ID NO:11:), (SEQ ID NO:12:) or (SEQ ID NO:13:), said (SEQ ID NO:13:) being a copy of the genomic sequence without or with introns or fragments of the introns with the prerequisite that it includes at least one of the exons or parts thereof (SEQ ID

NO:29:), (SEQ ID NO:30:), (SEQ ID NO:31:), (SEQ ID NO:33:), (SEQ ID NO:35:), (SEQ ID NO:35:), (SEQ ID NO:37:) or (SEQ ID NO:39:), fragments or any combination thereof having fragments of the introns (SEQ ID NO:32:), (SEQ ID NO:34:), (SEQ ID NO:36) or (SEQ ID NO:38) but still encoding a polypeptide having a functional activity substantially similar to that of human GFR α 4.

6. The use of nucleic acid sequence according to claims 1-5 for recording GFR α 4-mediated signalling in neurons or endocrine cells.

7. The use of nucleic acid sequence according to claims 6, wherein the endocrine cells are thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells or cells of pituitary intermediate lobe.

8. The use of nucleic acid sequence according to claim 1-5 for manufacturing polypeptides useful for diagnosing and/or treating tumours in parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma and pheochromocytoma, parathyroid hyperplasia, neuronal disorders or for preventing neuronal death or aberrant axonal sprouting.

9. A purified and isolated polypeptide, characterized in that it comprises a polypeptide having the amino acid sequence (SEQ ID NO:1:), (SEQ ID NO:2:), (SEQ ID NO:3:), (SEQ ID NO:4:), (SEQ ID NO:5:) or (SEQ ID NO:6:).

10. The polypeptide according to claim 9, characterized in that it has a structure substantially similar to an isoform of mouse GFR α 4.

11. The polypeptide according to claim 9, characterized in that it has a structure substantially similar to an isoform of human GFR α 4.

12. The use of the polypeptides according to claims 9-11 for determining GFR α 4-mediated signalling in neurons, endocrine cells, neoplasia, endocrine tumours, medullary thyroid carcinoma.

13. The use of the polypeptides according to claims 12, wherein the endocrine cells are thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells or cells of pituitary intermediate lobe.

14. The use of the polypeptides according to claims 12, wherein the medullary thyroid carcinoma comprises pheochromocytoma and parathyroid hyperplasia.

15. The use of polypeptides according to claims 9-11 for manufacturing a composition for diagnosing and/or treating neuronal disorders, osteoporosis, cancer in parathyroid gland cells, adrenal chromaffin cells and cells of pituitary intermediate lobe, in neoplasia, treatment of endocrine tumours, including medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia, for preventing axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures, preventing neuronal death without producing aberrant sprouting.

16. A method for obtaining a cell-line (clone) useful in the production of the transgenic non-human animal according to claims, characterized in that the genomic gene encoding a functional GFR α 4 receptor is inactivated by introducing into stem cells or cell lines (clones) a nucleic acid sequence or a part thereof, which sequence or part is capable of integrating to said genomic gene and by said integration functionally inactivates said genomic gene or makes it conditionally inactivatable.

17. A cell line (clone) obtainable by the method of claim 16,

c h a r a c t e r i z e d in that the cell-line (clone) is lacking a functional GFR α 4 receptor and producing altered levels of GFR α 4.

18. The use of the cell line (clone) of claim 17, c h a r a c t e r i z e d in that the cell lines are used for producing a transgenic non-human animal and/or diagnosing and/or treating osteoporosis, endocrine tumours, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia by recording GFR α 4-mediated signalling in endocrine cells, thyroid calcitonin-producing C-cells, parathyroid gland cells, adrenal chromaffin cells, cells of pituitary intermediate lobe, neoplasia, endocrine tumours, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia, neuronal disorders, for preventing axonal sprouting in epilepsy due to compensatory neurotrophic factor production after seizures or preventing neuronal death without producing aberrant sprouting.

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mGFR α 1 MFLATLYFVLPLDLLMSAEVSGGD.....RLDCVKASDQCLKEQSCSTKYRTLROCVAGK
 mGFR α 2 MILANAFLEFFFLDETLRSLASPSSPQGSSEL.HGWRPQVDCVRANELCAAESNCSSRYRTLROCLAGR
 mGFR α 3 MGLSWSPRPPLLMILLVLSLWLPLGAGNSLATENRFVNSCTQARKKCEANPAKKAAYQHLGSCSTSSL
 mGFR α 4 MAHCMESALLLLLLLGSASFT.....
 cGFR α 4 MRGILYFCTLILLEGMAEAVSSSR.....DCLQAGESCTNDPICSSKFRTLROCIAGN

mGFR α 1ETNFSLTSGLEAKDECRSAMEALKQKSLYNCRCKRGMKKEKNCLRIYWSMYQSL.QGN
 mGFR α 2DRNTML.....ANKECQAALVLEQESPLYDCRCKRGMKKEKLCCLQIYWSIHGLTEGE
 mGFR α 3SRPLPLEESAMSAD.CLEAAEQLRNSSLIDCRCHRRMKHQATCLDIYWTVHPARSLGD
 mGFR α 4
 cGFR α 4GAN...KLGPDANKQCRSTVTALLSSQLYGCKCKRGMKKEKHCLSVYWSIHHTLMEGM

mGFR α 1 DLLEDSPYEPVNSRLSDIFRAVPFISDVQQVEHISKGNCLDAAKACNLDDTCKKYRSAYITPCTTS
 mGFR α 2 EFYEASPYEPTVTSRLSDIFRLASIFSGTGADPVVSAKSNHCLDAAKACNLNDNCKKLRSYISICNRE
 mGFR α 3 YELDVSPYEDTIVTSKPW.....KMNLSKLNMLKPDSDLCLKFAMLC TLHDKCDRLRKAYGEACSG.
 mGFR α 4DGNRCVDAAEACTADERCQQLRSEYVARCLGR
 cGFR α 4 NVLESSPYEPF.IRGFDYVRLASI...TAGSENEVTQVNRCLDAAKACNVDEMQRRLRTEYVSFCIRR

mGFR α 1 MS.NEV.....CNRRKCHKALRQFFDKVPAKHSYGMLFCSC..RDVACTERRRQTIVPVCSYEER
 mGFR α 2 ISPTER.....CNRRKCHKALRQFFDRVPSEYTYRMLFCSC..QDQACAERRRQTILPSCSYEDK
 mGFR α 3IRCQRHLCLAQLRSFFEKAAESHAQGLLCPCAPEDAGCGERRRNTIAPSCALPS.
 mGFR α 4 AAPGGRPGPGGCVRSRRCRRALRRFFARGPPALTHALLECGC..EGSACAERRRQTIFAPACAFSGP
 cGFR α 4 LARADT.....CNRSKCHKALRKFFDRVPPEYTHELLECPC..EDTACAERRRQTIVPACSYESK

mGFR α 1ERPNCNLQDSCKTNYICRSRLADFFTNCPESRSVSN
 mGFR α 2EKPNCCLDLRSLCRTDHLRSRLADFHANCRASTYRTITS
 mGFR α 3VTPNCCLDLRSFCRADPLCRSRLMDFQTHCHPMD.ILGT
 mGFR α 4GLVPPSCLEPLERCERSRLCRPRLAFQASCAPAPGSRDR
 cGFR α 4EKPNCCLAPLDSCRENYVCRSRYAEFQFNCQPSLQTASG

mGFR α 1 CLKENYADCLLAYSGLIGTVMTPNYIDS..SSLSVAPWCDSCNSGNDLEDCLKFLNFFKDNTCLKN
 mGFR α 2 CPADNYQACLGSYAGMIGFDMTPNYVDSNPTGIVVSPWCNCRGSGNMEECEKFLKDFTENPCLRN
 mGFR α 3 CATEQ.SRCLRAYLGLIGTAMTPNFISKVNTTVALS..CTCRGSGNLQDECEQLERSFSQNPCLVE
 mGFR α 4 CPEEGGPRCLRVYAGLIGTVVTPNYLDN..VSARVAPWCGCAASGNRREECEAFRKLFTRNPCLDG
 cGFR α 4 CRRDSYAACLLAYTGIIGSPITPNYIDNSTSS..IAPWCTCNASGNRQEECESFLHLFTDNVCLQN

mGFR α 1 AIQAFNGSDVTMWQPAPPVQTTTAMTTTAFRIKKNPLGP..AGSENEIPTHVLPPCANLQAQKLK
 mGFR α 2 AIQAFNGTVDNM.SPKGP...TFSATQAPRVEKTPSLPDDLSDESTSLGTSVITTCSTSIQEQGLK
 mGFR α 3 AIAA.....
 mGFR α 4 AIQAE.....
 cGFR α 4 AIQAFNGTYLN..AATAPSIPTTQMYKQERNANRAAATLSENI FEHL.....

mGFR α 1 SNVSGSTHCLSDNDYGKDLGAGASSHITTKSMAAPPSGGLSSLPVMVFTALAALLSVSLAETS
 mGFR α 2 ANNSKELSMCFTELTTNISPGSKKVIKLYSGSCRAR.....LSTALTALPLLMVTLA
 mGFR α 3KMRFHRLQFSQDWADSTFSVVQQQNSNPALRLQPRLPILSFSILPLILLQTLW
 mGFR α 4DSLQPSVLQDQTAGCCFPR..VSWLYALTALALQALL
 cGFR α 4QPTKVAGEERLLRGSTRLSSETSSPAAPCHQAASLLQLWLPPTLAVLSHFMM

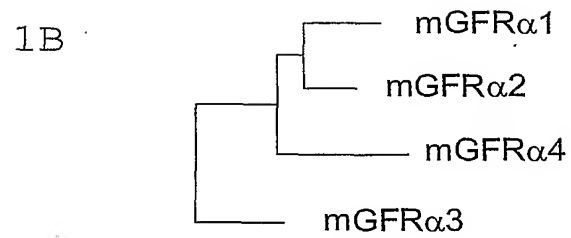
D1

D2

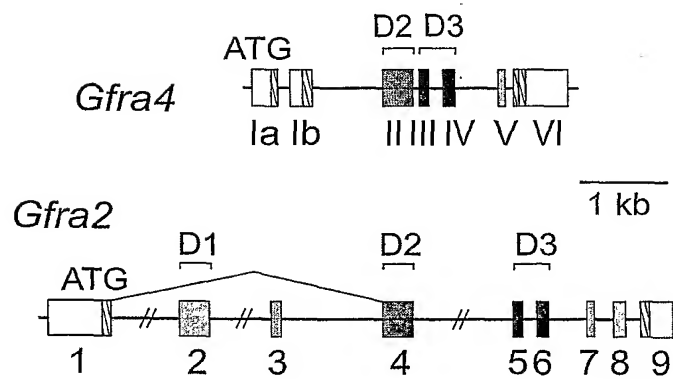
D3

Fig. 1A

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1C



1D

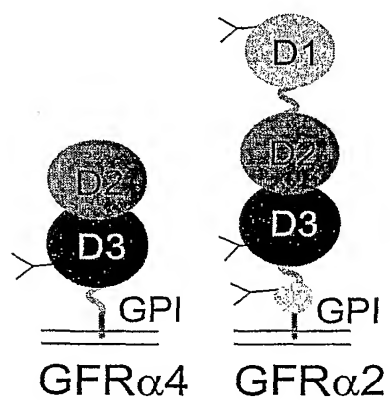


Fig. 1B-D

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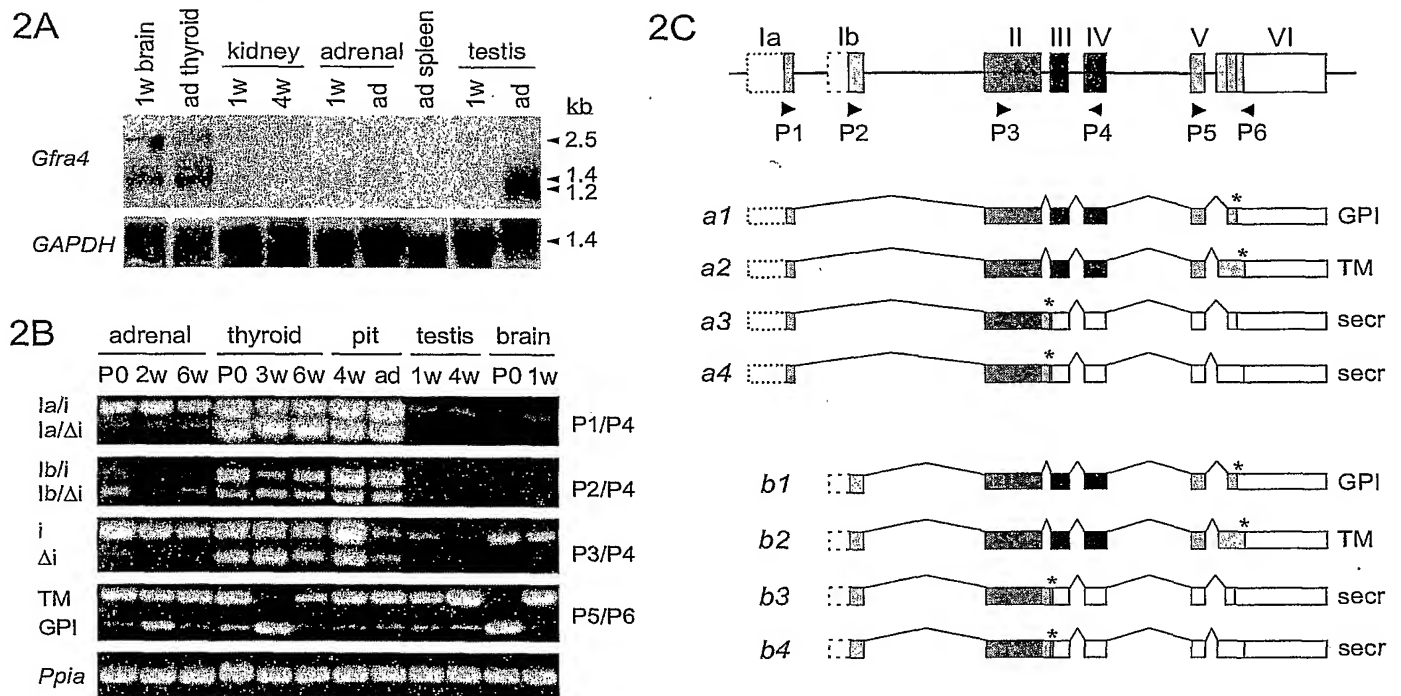


Fig. 2

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exon.AGCTragt.....intron....tyyyyyyyyyncaG...exon
Ia...GGTgaga....1284 bp....ccgcccttcaccagG...II
Ib...AGCTaaga.....851 bp....ccgcccttcaccagG...II
II...CGTgcgt.....53 bp....ccgggcgcgcgcagG...III
III..AGCTaggc.....81 bp....ggggtccccgcagG...IV
IV...GGTgagg.....541 bp....tctgcactccgcagA...V
V....GGTtaggt.....140 bp....tcctaacgcccaagG..VIa
V....GGTtaggt.....73 bp....cttacccttcctagG..VIb

Fig. 3

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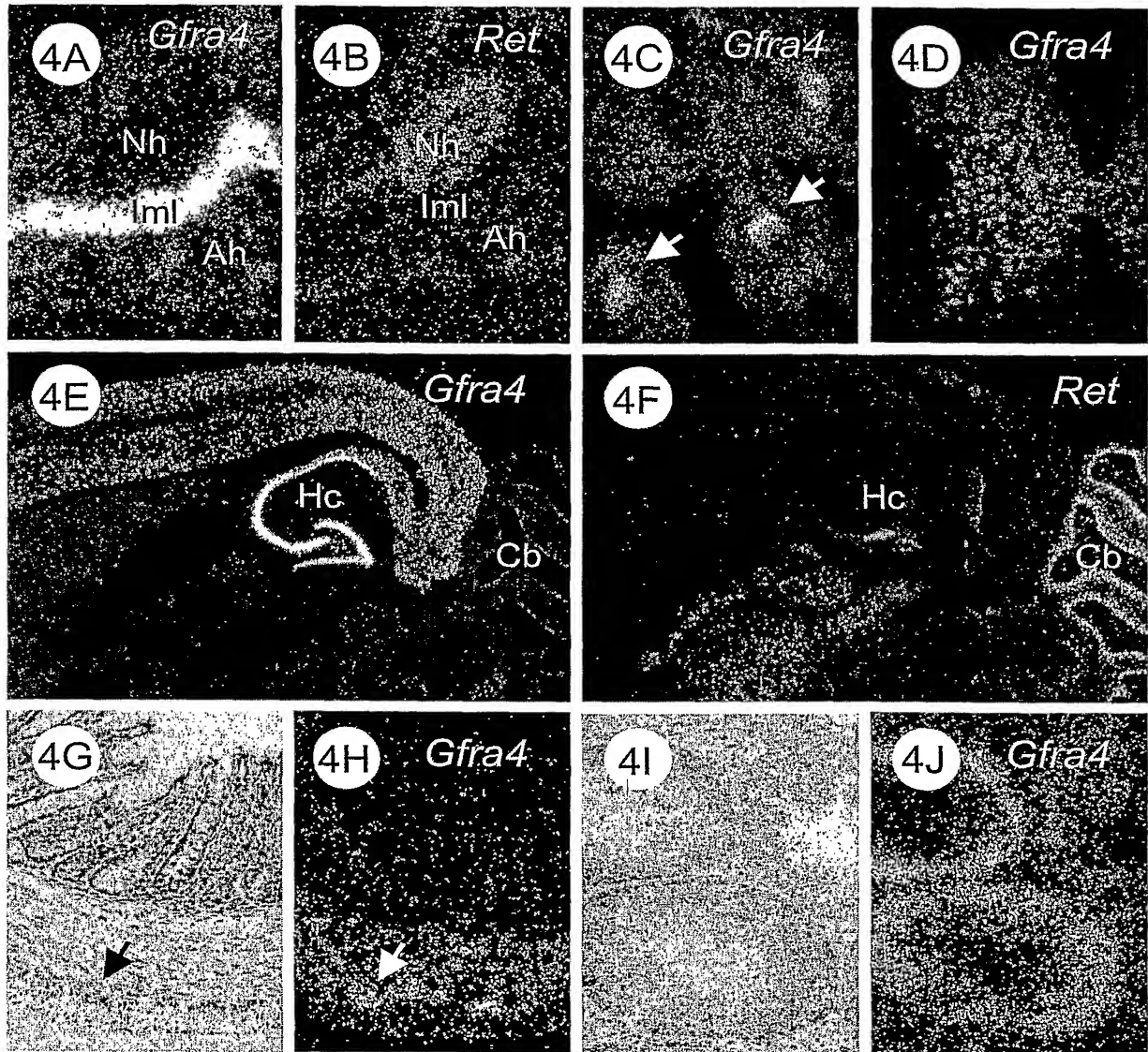


Fig. 4

6/27

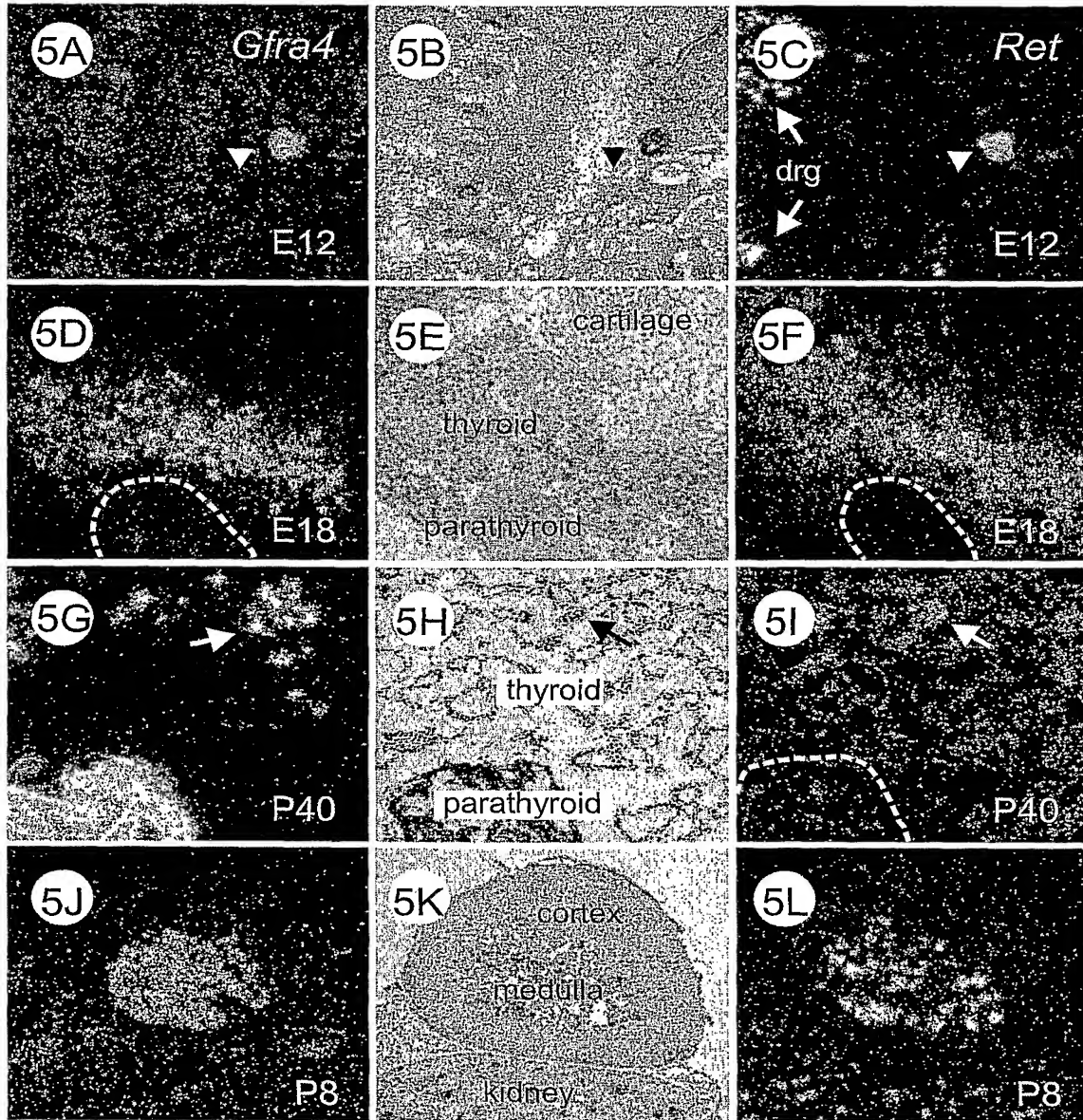


Fig. 5

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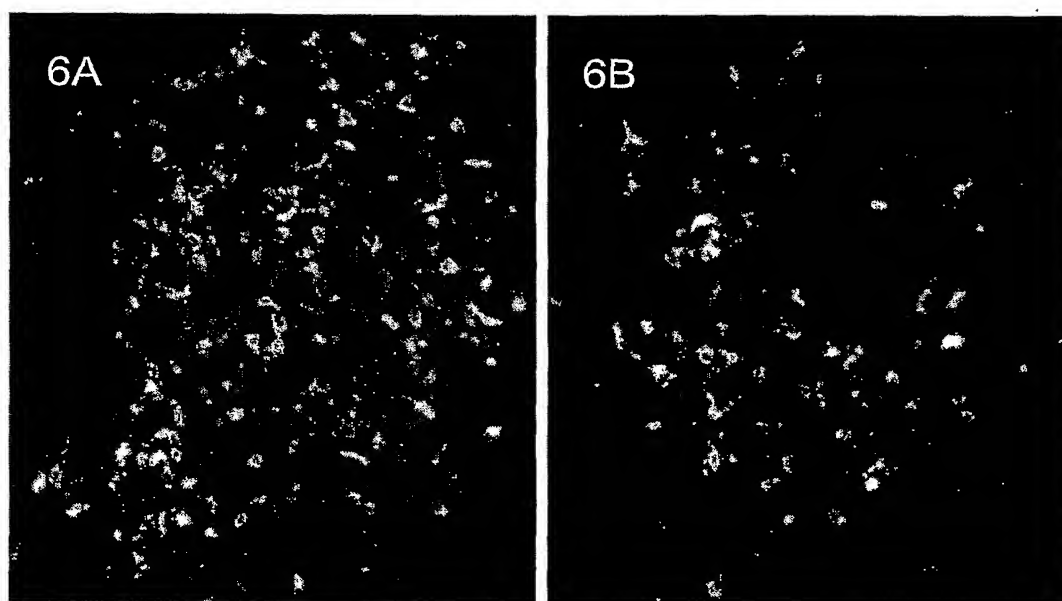


Fig. 6

8/27

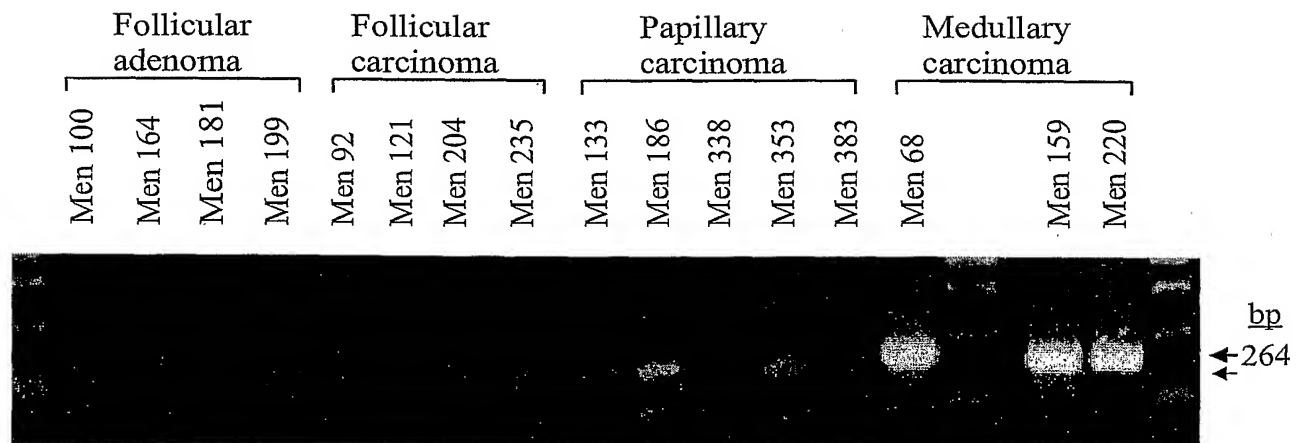


Fig. 7

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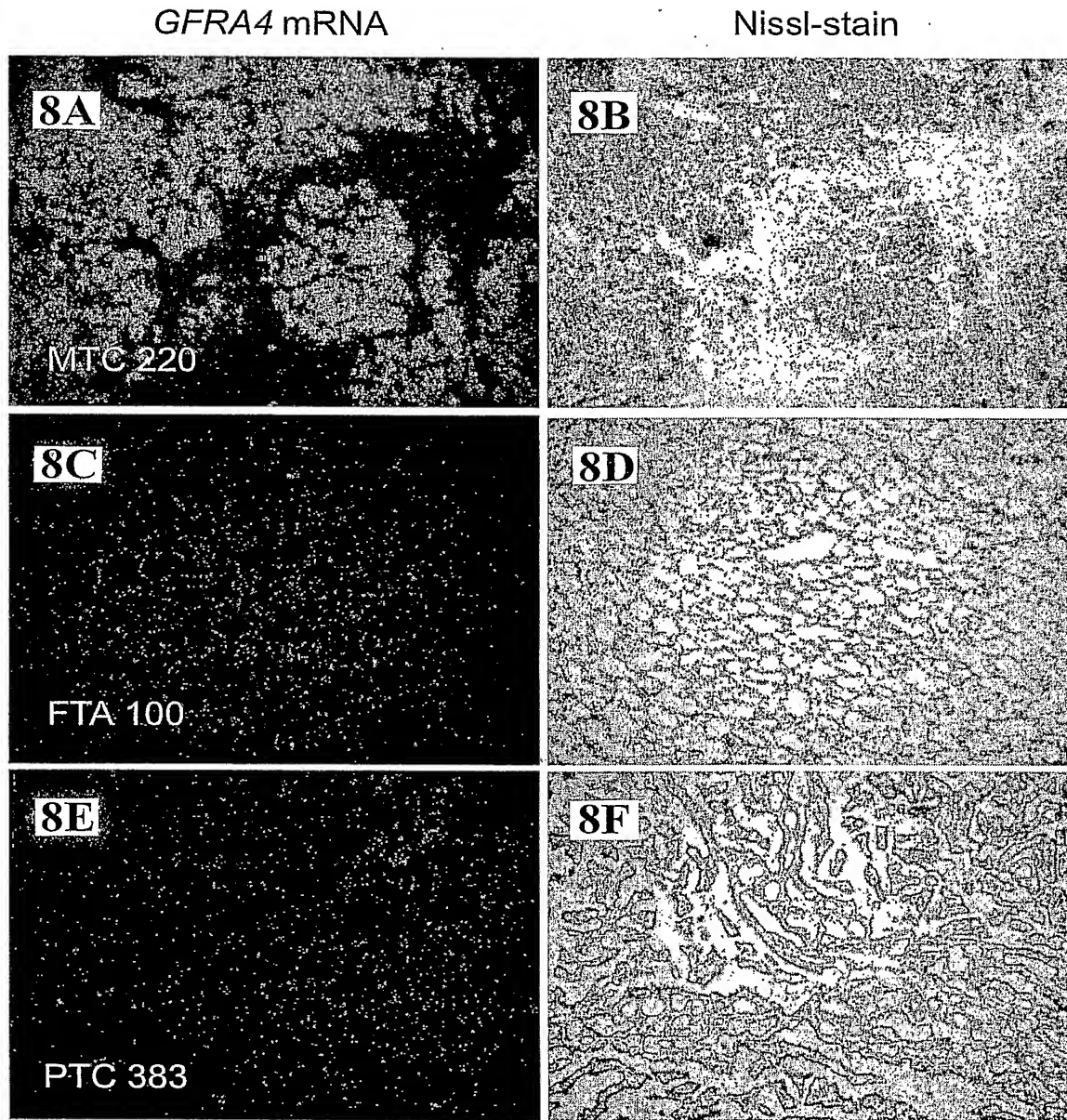


Fig. 8

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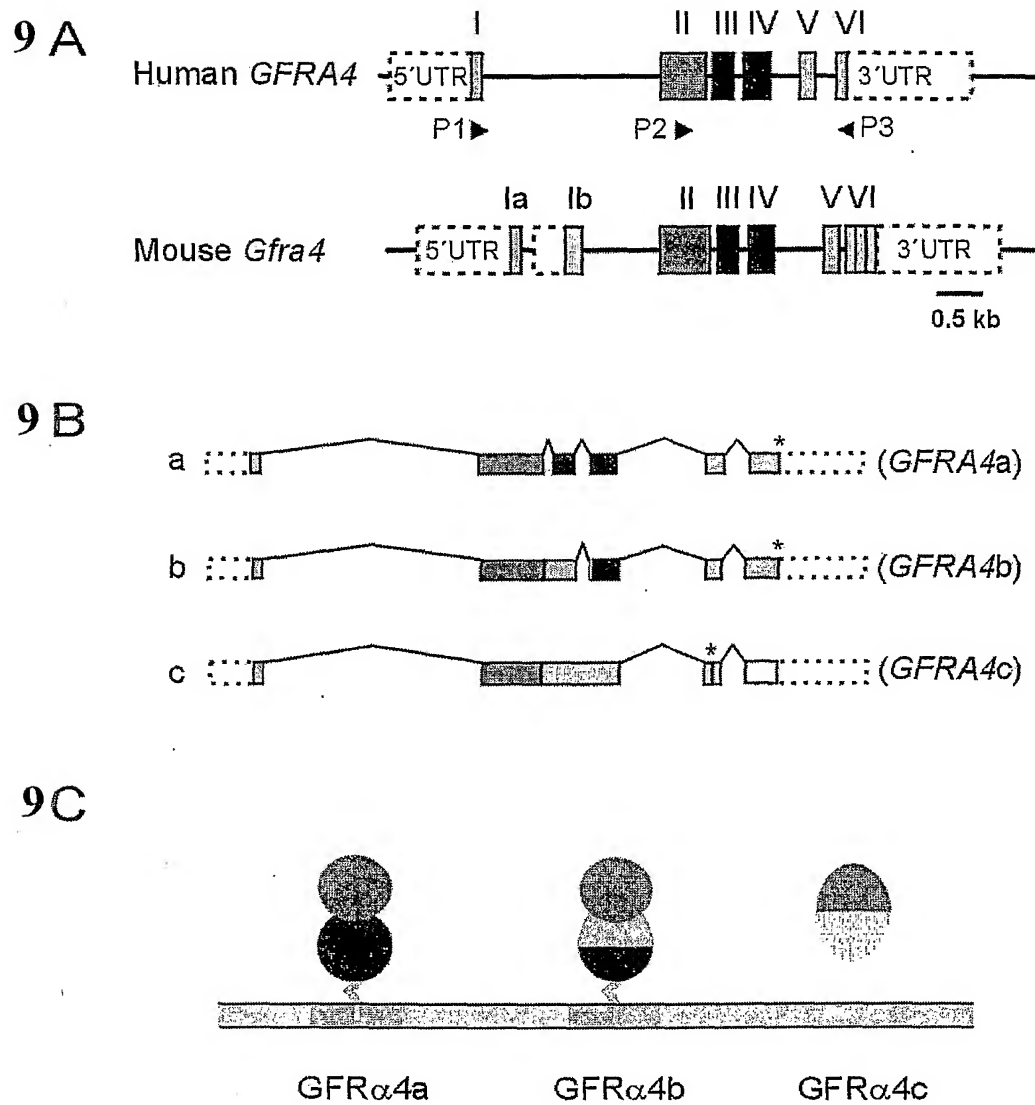


Fig. 9

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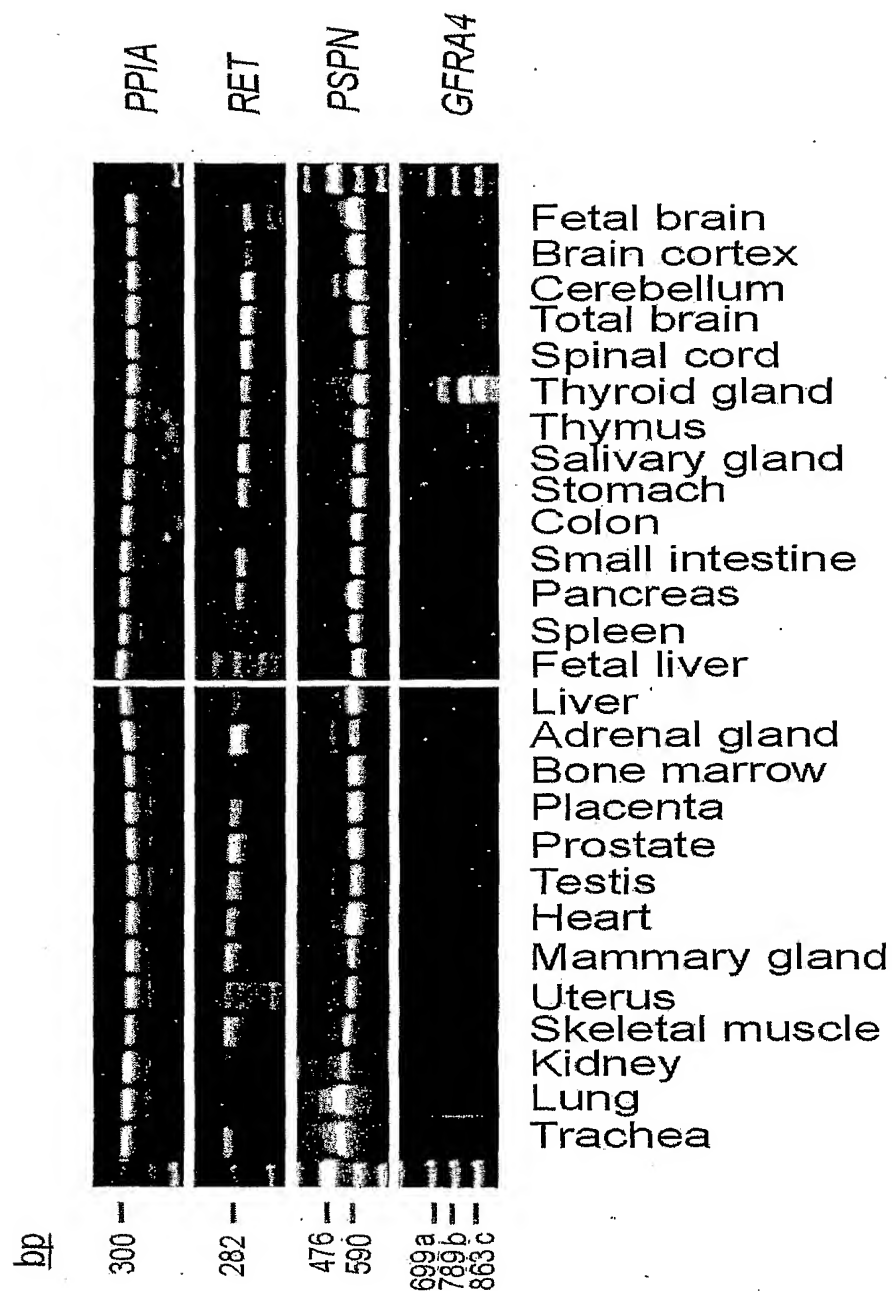


Fig. 10

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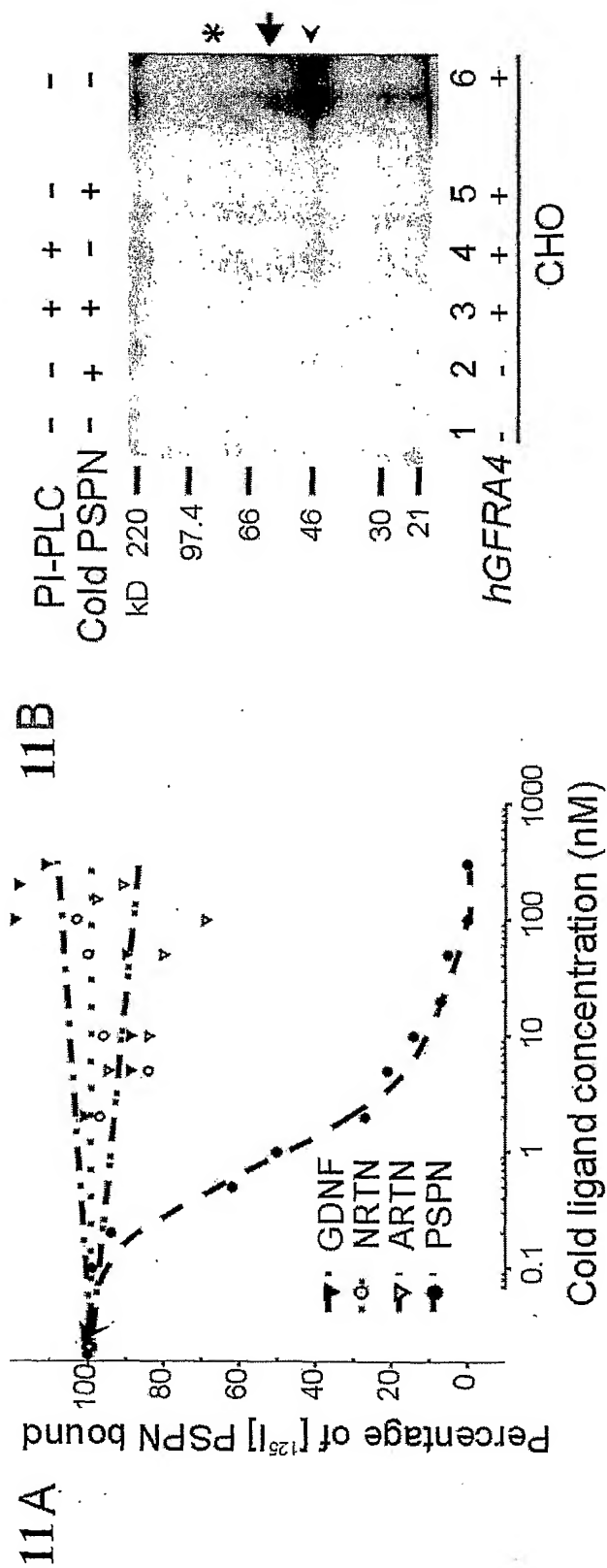
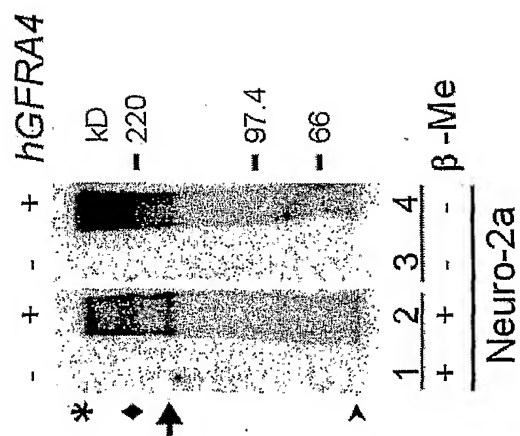
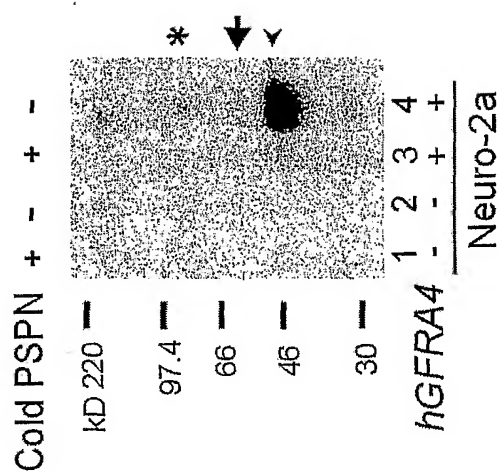


Fig. 11 A-B



110



11C

Fig. 11 C-D

11F

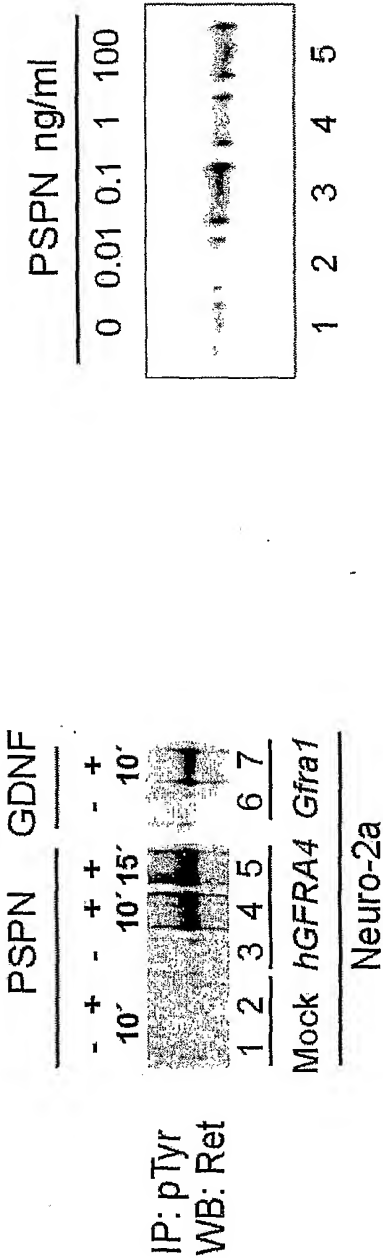


Fig. 11 E-E

11E

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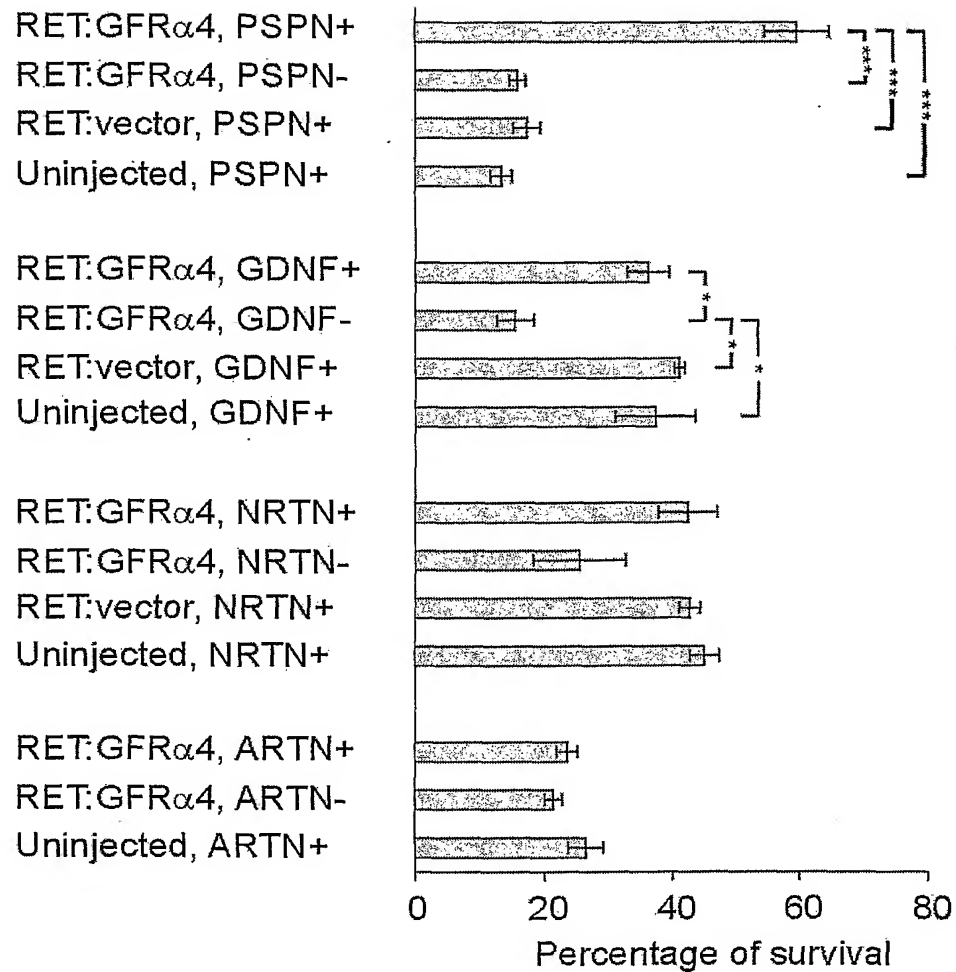


Fig. 12

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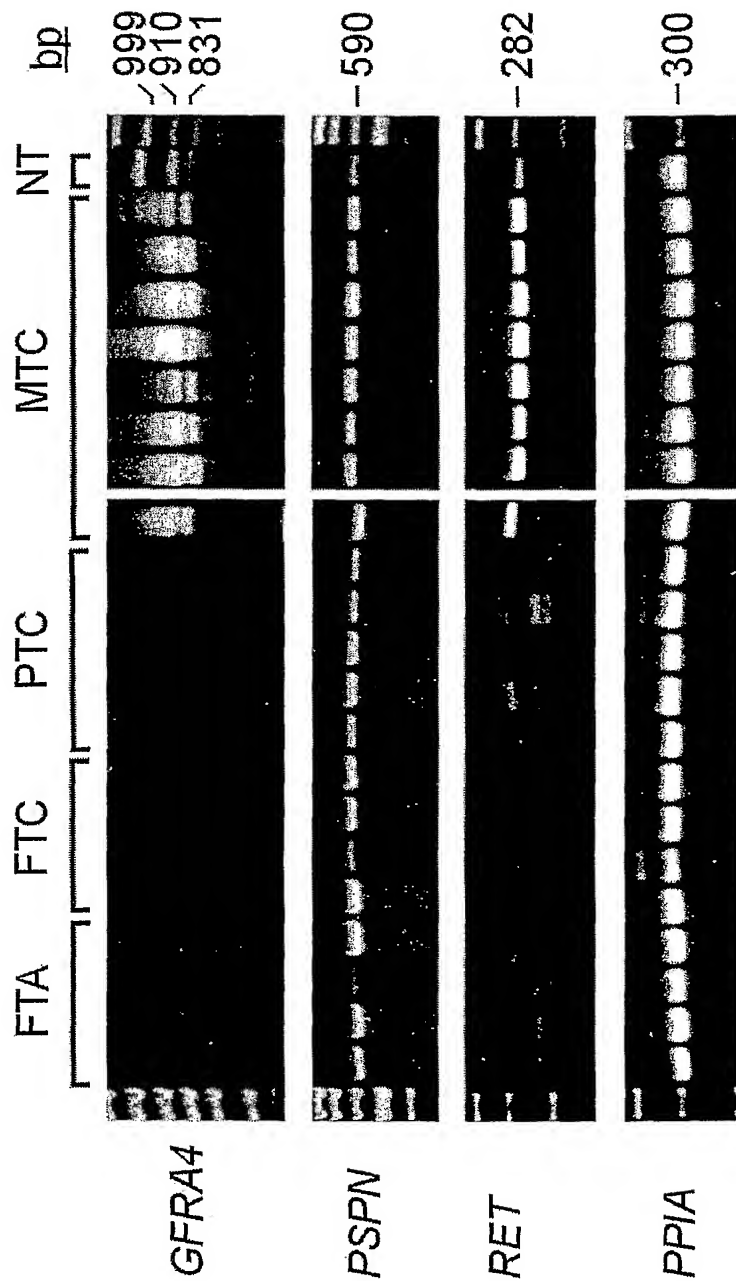


Fig. 13

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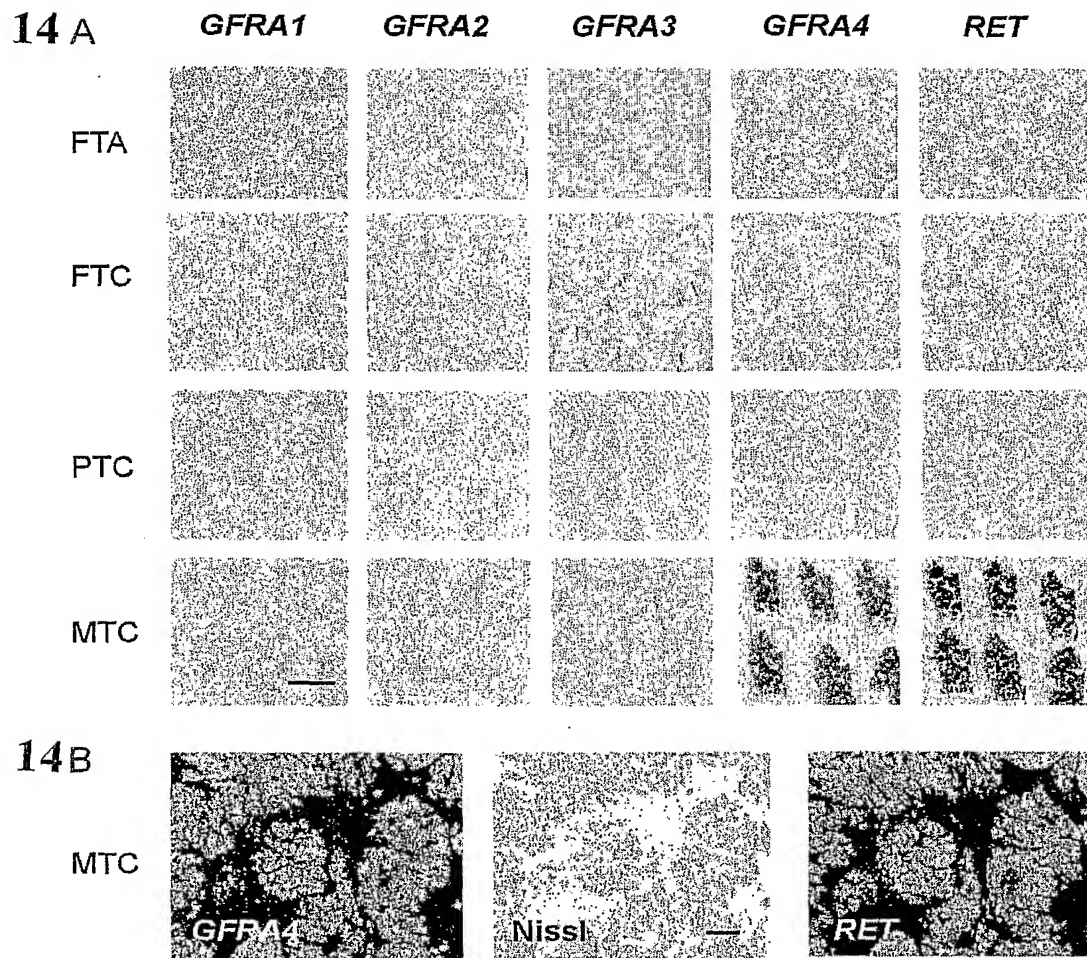
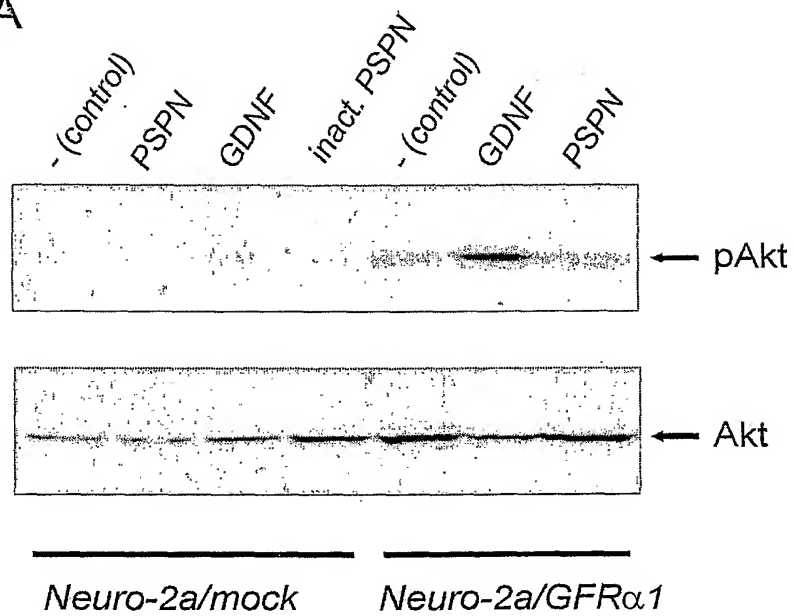


Fig. 14

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15A



15B

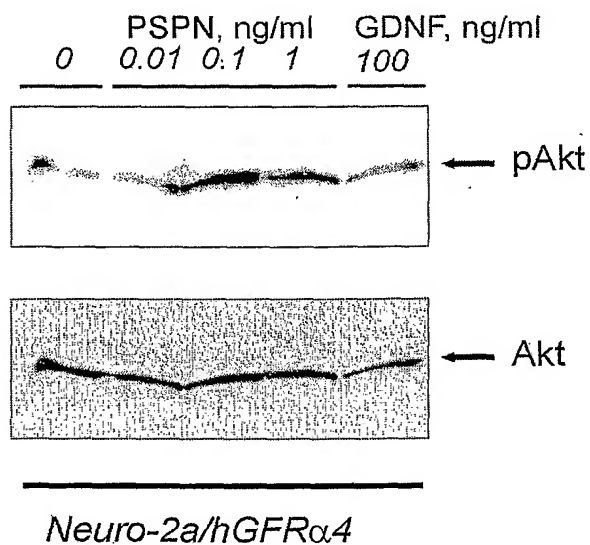
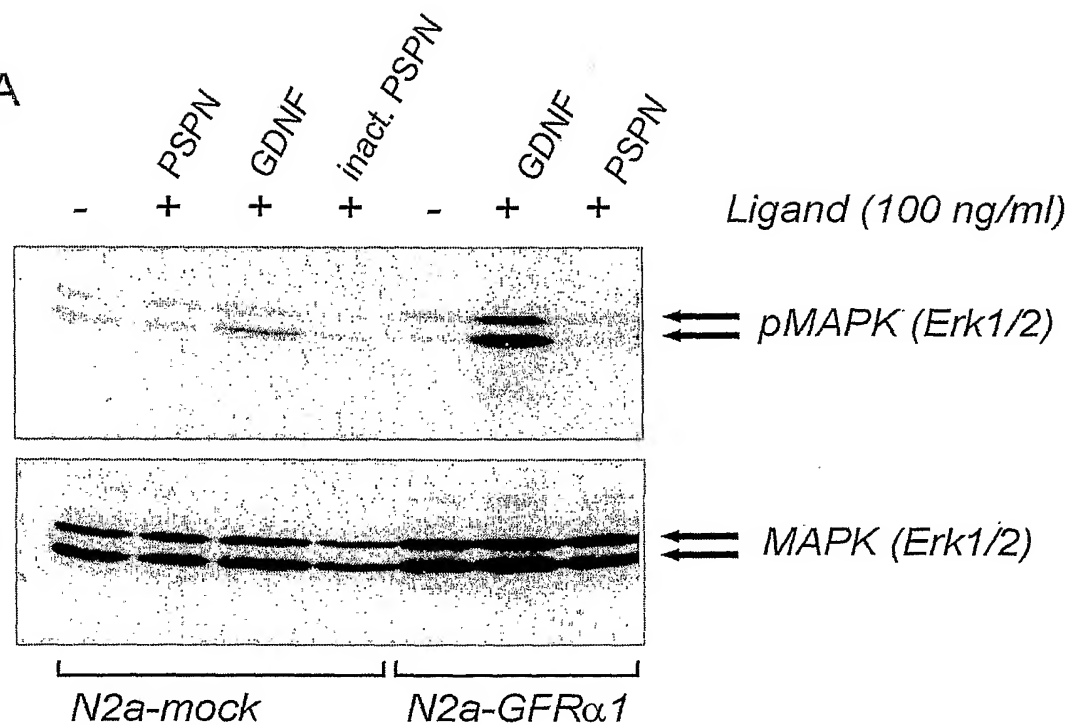


Fig. 15

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16 A



16 B

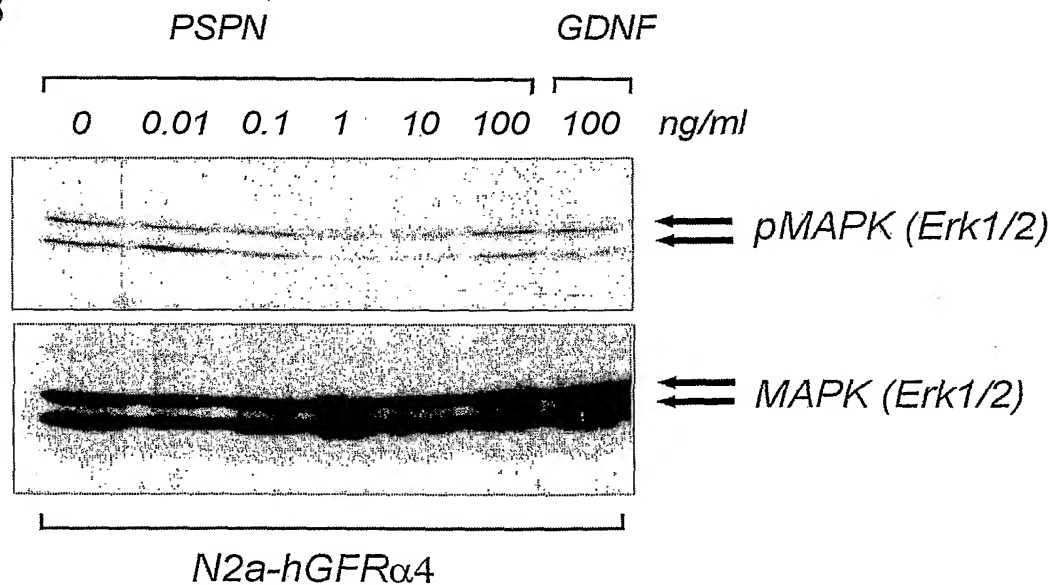
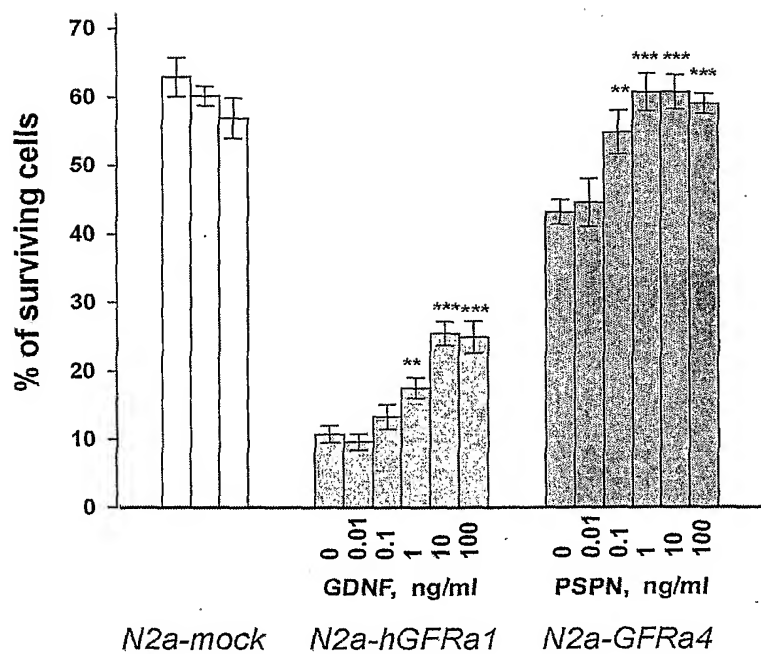


Fig. 16

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17A



17B

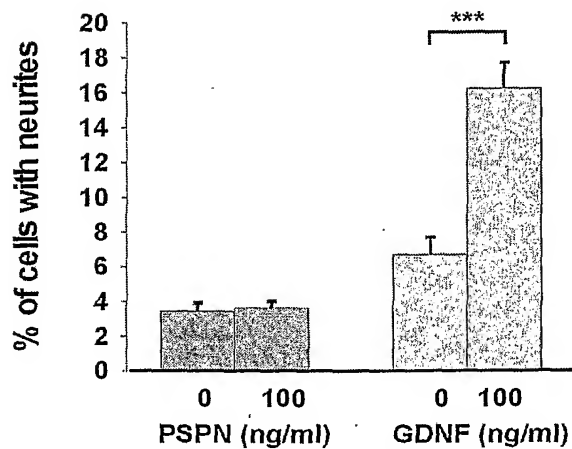


Fig. 17

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Fig. 18A

Mouse Gfra4-[GPI]a1 cDNA sequence, length: 783

```

1  atggcccaact gcatggagtc tgcactgctg ctgttgttgc tgctggggtc
51  tgcgagcttt accgacggga atcgctgcgt ggacgcggcc gaggcgtgta
101 cagcagacga gcggtgccag cagctgcgct ctgagtacgt ggcacgatgc
151 ctgggccggg cagcgcccg gggcaggccg ggaccgggg gctgcgtgcg
201 ctcccgtgc cgccgagccc tgcgccgctt cttcgcgcgt gggcctccgg
251 cgctcacgca tgcgctgctc ttctgcggct gcgaaggctc cgcgtgcgcc
301 gagcgccggc gccagacttt cgcgcccgcc tgcgcgttct ccggcccggg
351 gttggtgccg cctcttgcc tggagcccct ggagcgctgc gagcgcgacc
401 gcctgtgccg gcccgcgtct cttgccttcc aggcctcatg cgctcccgcg
451 cccggctccc gcgaccgctg cccggaggag gggggcccg gtgtgtctgcg
501 cgtctacgca ggctcatag gcaccgtggt caccccaac tacctggaca
551 acgtgagcgc gcgcgttgcg ccctggtgcg gctgtgcggc cagtggaaac
601 cggcgcgaaag aatgcgaagc cttccgcaag ctctttacaa ggaaccctg
651 cttggatggt gccatacaag cctttgacag cttgcagcca tcagttctgc
701 aggaccagac tgctgggtgc tgtttcccgc ggggtgcctg gctgtatgca
751 ctcaactgcc tggctctcca ggccctgctc tga

```

Fig. 18B

Mouse GFR α 4 GPI-linked isoform a1 amino-acid sequence, length: 261

```

1  MAHCMESALL LLLLLGSASF TDGNRCVDAA EACTADERCQ QLRSEYVARC
51  LGRAAPGGRP GPGGCVRSRC RRALRRFFAR GPPALTHALL FCGCEGSACA
101 ERRRQTFAPA CAFSGPGLVP PSCLEPLERC ERSRLCRPRL LAFQASCAPA
151 PGSRDRCPEE GGPRCLRVYA GLIGTVVTPN YLDNVSARVA PWC GCAASGN
201 RREECEAFRK LFTRNPCLDG AIQAFDSLQP SVLQDQTAGC CFPRVSWLYA
251 LTALALQALL*

```

Fig. 18

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Fig. 19A

Mouse Gfra4 transcript a2 cDNA sequence, length:882

```

1  atggcccaact gcatggagtc tgcactgctg ctgttggtgc tgctgggggc
51  tgcgagcttt accgacggga atcgctgcgt ggacgcggcc gaggcgtgta
101 cagcagacga gcggtgccag cagctgcgct ctgagtacgt ggcacgatgc
151 ctgggceggg cagcgcccgg gggcaggccg ggacccgggg gctgcgtgcg
201 ctcccgtctg cgccgagccc tgcgccgctt cttcgcgcgt gggcctccgg
251 cgctcacgca tgcgtgctc ttctgcggct gcgaaggctc cgcgtgcgcc
301 gagcgccggc gccagacttt cgcgcccgcc tgcgcgttct ccggcccggg
351 gttggtgccg cctcttgcc tggagcccct ggagcgctgc gagcgagcc
401 gcctgtgccg gcccgtctc cttgccttcc aggcctcatg cgctcccgcg
451 cccggctccc gcgaccgctg cccggaggag gggggcccgc gttgtctgcg
501 cgtctacgca ggcctcatag gcaccgtggt caccccaac tacctggaca
551 acgtgagcgc gcgcgttgcg ccctggtgcg gctgtgcggc cagtggaaac
601 cggcgcgaa aatgcgaagc cttccgcaag ctctttacaa ggaaccctg
651 cttggatggt gccatacaag ctttgacag cttgcagcca tcagttctgc
701 aggaccagac tgctgggtgc tgtttcccgc gggcaaggca cgagtggcct
751 gagaagagct ggaggcagaa acagtccttg ttttgccta acgccaagg
801 tgtcctggct gtatgcactc actgccctgg ctctccaggc cctgctctga
851 ttaggaacat gaaccgtgga cgacacagct ga

```

Fig. 19B

Mouse GFR α 4 putative TM-isoform a2 amino-acid sequence, length:294

```

1  MAHCMESALL LLLLLGSASF TDGNRCVDAA EACTADERCQ QLRSEYVARC
51  LGRAAPGGRP GPGGCVRSRC RRALRRFFAR GPPALTHALL FCGCEGSACA
101 ERRRQTFAPA CAFSGPGLVP PSCLPLERC ERSRLCRPRL LAFQASCAPA
151 PGSRDRCPEE GGPRCLRVYA GLIGTVVTPN YLDNVSARVA PWC GCAASGN
201 RREECEAFRK LFTRNPCLDG AIQAFDSLQP SVLQDQTAGC CFPRARHEWP
251 EKSWRQKQSL FCPNAQGVLA VTHCPGSPG PALIRNMNRG RHS*

```

Fig. 19

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Fig. 20A

Mouse Gfra4 transcript a3/a4 cDNA sequence, length:573

```

1  ATGGCCCACT GCATGGAGTC TGCACGTGCTG CTGTTGTTGC TGCTGGGGTC
51  TGCGAGCTTT ACCGACGGGA ATCGCTGCGT GGACGCGGCC GAGGCGTGTA
101 CAGCAGACGA GCGGTGCCAG CAGCTGCGCT CTGAGTACGT GGCACGATGC
151 CTGGGCCGGG CAGCGCCCGG GGCAGGCCG GGACCCGGGG GCTGCGTGCG
201 CTCCCGCTGC CGCCGAGCCC TCGCGCGCTT CTTCGCGCGT GGGCCTCCGG
251 CGCTCACGCA TCGCTGCTC TTCTGCGGCT GCGAAGGCTC CGCGTGCGCC
301 GAGCGCCGGC GCCAGACTTT CGCGCCCGCC TCGCGCTTCT CCGGCCCGGG
351 GTTGGTGCCG CCCTCTTGCC TGGAGCCCCT GGAGCGCTGC GAGCGCAGCC
401 GCCTGTGCCG gtgcggtgct gcggggcggg ctggggcgct caccgcgctc
451 cgggcgcgcg cagGCCCCGT CTCCTTGCC TCCAGGCCTC ATGCGCTCCC
501 GCGCCCGGCT CCCGCGACCG CTGCCCCGAG GAGGGGGGCC CGCGTTGTCT
551 GCGCGTCTAC GCAGGCCTCA TAG

```

Fig. 20B

Mouse GFR α 4 soluble isoform a3/4 amino-acid sequence, length: 191

```

1  MAHCMESALL LLLLLGSASF TDGNRCVDAA EACTADERCQ QLRSEYVARC
51  LGRAAPGGRP GPGGCVRSRC RRALRRFFAR GPPALTHALL FCGCEGSACA
101 ERRRQTFAPA CAFSGPGLVP PSCLEPLERC ERSRLCRCVR AGRAGPLTRV
151 RARAGPVSLP SRPHALPRPA PATAARRRG RVVCASTQAS*

```

Fig. 20

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Fig. 21A

Human *GFRA4a* cDNA sequence (GPI-linked splice form a), length: 810

```

1  ATGGTCCGCT GCCTGGGGCC TGCCTGCTG CTGCTGCTGT TACTGGGGTC
51  GGCGAGCTCG GTCGGAGGGA ACCGATGTGT GGACGCGGCC GAAGCCTGCA
101 CGGCGGACGC GCGGTGCCAG CGTTTGCGCT CCGAGTATGT GGCGCAGTGC
151 CTGGGCCGGG CTGCGCAGGG GGGCTGTCCC CGCGCCCGCT GCCGCCGGGC
201 CCTGCGCCGC TTCTTCGCCC GCGGGCCGCC CGCGCTCACC CACGCACTGC
251 TCTTCTGCCC GTGCGCGGGC CCCGCGTGCG CCGAGCGTCG GCGCCAGACC
301 TTCGTGCCCT CCTGCGCCTT TTCGGGGCCC GGCCCGCGCG CGCCCTCCTG
351 CCTTGAGCCC TTAAACTTCT GCGAGCGCAG CCGGGTCTGC AGGCCTCGCC
401 TCCTGGCCTT TCAGGTCTCG TGACCCCCAG CGCCAGCGCG CCCCAGCGGC
451 TGCCTGCTGG ACCAGGGCGC CCGCTGCCTG CGCGCCTACG CGGGCCTCGT
501 GGGCACC GCC GTACCCCTA ACTACGTGGA CAACGTGAGC GCGCGCGTGG
551 CGCCCTGGTG CGACTGCGGA GCCAGCGGGA ACCGGCGTGA GGA CTGCGAA
601 GCCTTCCGGG GGCTCTTTAC CAGGAACCGC TGCTTGATG GTGCCATTCA
651 GGCCTTTGCC AGCGGGTGCC CCCCAGTCCT GCTGGACCAG CTGAACCCCC
701 AGGGAGACCC GGAGCACAGC CTCCTGCAGG TGCCTCCAC AGGCAGGGCC
751 CTGGAGAGAC GCTCCCTGCT CTCCATACTT CCTGTCCTGG CTCTCCGGC
801 CCTGCTCTGA

```

Fig. 21B

Human *GFRA4a* amino-acid sequence (GPI-linked isoform a), length: 269

```

1  MVRCLGPALL LLLLLGSASS VGNRCVDAA EACTADARCQ RLRSEYVAQC
51  LGRAAQGGCP RARCRRALRR FFARGPPALT HALLEFCPCAG PACAERRRQT
101 FVPSCAFSGP GPAPPSCLEP LNFERSRVC RPRLLAQFVS CTPAPSAPDG
151 CLLDQGARCL RAYAGLVGTA VTPNYVDNVS ARVAPWDCG ASGNRREDCE
201 AFRGLFTRNR CLDGAIQAF A SGWPPVLLDQ LNPQGDPEHS LLQVSSTGRA
251 LERRSLLSIL PVLALPALL*

```

Fig. 21

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Fig. 22A

Human GFRA4b splice form cDNA sequence, length: 900

ATGGTCCGCTGCCTGGGGCCTGCGCTGCTGCTGCTGCTGTTACTGGGGTCGGCGAGCTCGGTCCGAGGG
 AACCGATGTGTGGACGCGGCCGAAGCCTGCACGGCGGACGCGCGGTGCCAGCGTTTTCGCTCCGAGTAT
 GTGGCGCAGTGCCCTGGGCGGGCTGCGCAGGGGGGTGTCCCCGCGCCCGCTGCCGCGGGGCCCTGCGC
 CGCTTCTTCGCCCCGCGGGCCGCCGCGCTACCCACGCACTGCTCTTCTGCCCGTGCGCGGGCCCCGCG
 TCGCCCCGAGCGTCGGCGCCAGACCTTCGTGCCCTCCTGCGCCTTTTCGGGGCCCCGGCCCCGCGCCGCC
 TCCTGCCTTGAGCCCTTAAACTTCTGCGAGCGCAGCCGGGTCTGCAGGTGCGCGCGGGCGGCGGGG
 CCGTGGCGAGGGTGGGGACGGGGCCTCTCTCCGGCTCACCGCCCTCCCGCCGCGCAGGCCTCGCCTCCT
 GGCCTTTTCAGGTCTCGTGCACCCAGCGCCAGCGCCCCGACGGCTGCCTGCTGGACCAGGGCGCCCG
 CTGCCTGCGCGCTACGCGGGCCTCGTGGggtccccgcaggcaccgcggtcaccctaaactacgtggac
 aacgtgagcgcgcgcggtggcgccctggtgcgactgaggagccagcggaaccggcggtgaggactgcgaa
 gccttcggggggctctttaccaggaaccgctgcttggATGGTGCCATTCAGGCCTTTGCCAGCGGGTGG
 CCCCCAGTCCTGCTGGACCAGCTGAACCCCCAGGGAGACCCGGAGCACAGCCTCCTGCAGGTGTCCTCC
 ACAGGCAGGGCCCTGGAGAGACGCTCCCTGCTCTCCATACTTCTGTCTGCTGCTCTCCCGGCCCTGCTC
 TGA

Fig. 22B

Human GFR α 4b (putative GPI-anchored isoform b) amino-acid sequence, length:299

The non-homologous part (from GFR α 4a) is indicated in grey, this corresponds to the translated intron 2, exon 3 (in other frame), and four amino acids (GVPA) from intron 3.

MVRCLGPALLLLLLLLGSASSVGGNRCVDAAEACTADARCQRLRSEYVAQCLGRAAQGGCPRARCRRALR
 RFFARGPPALTHALLFCPCAGPACAERRRQTFVPSCAFSGPGPAPPSCLEPLNFCERSRVCR CARAAAG
PWRGWGRGLSPAHRPPAAQASPPGLSGLVHPSAQRPRRLPAGPGRPLPARLRGPRGVPAGTAVTPNYVD
 NVSARVAPWCDCGASGNRREDCEAFRGLFTRNRCLDGAIQAFASGWPPVLLDQLNFPQGDPEHSLQVSS
 TGRALERRSLLSILPVLALPALL*

Fig. 22

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Fig. 23A

Human *GFRA4c* (putative soluble form) cDNA sequence, length: 549

```

1  ATGGTCCGCT GCCTGGGGCC TGCGCTGCTG CTGCTGCTGT TACTGGGGTC
51  GGCGAGCTCG GTCGGAGGGA ACCGATGTGT GGACGCGGCC GAAGCCTGCA
101 CGGCGGACGC GCGGTGCCAG CGTTTGCGCT CCGAGTATGT GGCAGCTGC
151 CTGGGCCGGG CTGCGCAGGG GGGCTGTCCC CGCGCCCGCT GCCGCCGGGC
201 CCTGCGCCGC TTCTTCGCCC GCGGGCCGCC CGCGCTCACC CACGCACTGC
251 TCTTCTGCCC GTGCGCGGGC CCCGCGTGCG CCGAGCGTCG GCGCCAGACC
301 TTCGTGCCCT CCTGCGCCTT TTCGGGGCCC GGCCCCGCGC CGCCCTCCTG
351 CCTTGAGCCC TTAAACTTCT GCGAGCGCAG CCGGGTCTGC AGGCCTCGCC
401 TCCTGGCCTT TCAGGTCTCG TGCACCCAG CGCCCAGCGC CCCCAGCGGC
451 TGCCTGCTGG ACCAGGGCGC CCGCTGCCTG CGCGCCTACG CGGGCCTCGT
501 GGGGTCCCCG CAGGCACCGC CGTCACCCCT AACTACGTGG ACAACCTGA

```

Fig. 23B

Human *GFRα4c* (putative soluble isoform c) amino-acid sequence, length: 182

```

1  MVRCLGPALL LLLLLGSASS VGNRCVDAA EACTADARCQ RLRSEYVAQC
51  LGRAAQGGCP RARCRRALRR FFARGPPALT HALLFCPCAG PACAERRRQT
101 FVPSCAFSGP GPAPPSCLEP LNFCSRSRVC RPRLAFQVS CTPAPSAPDG
151 CLLDQGARCL RAYAGLVGSP QAPPSPLTTW TT*

```

Fig. 23

27/27

Human *GFRA4* locus genomic sequence

..CTATCAGACTAGGGCTCTGCCAGCCATCCTTCTCTGTTGAAGGTCCAGC**ATGGT**CCGCTGCCTGG
GGCCTGCGCTGCTGCTGCTGCTGTTACTGG**gt**..longintron..**agGGT**CGGCGAGCTCG
GTCGGAGGGAACCGATGTGTGGACGCGGCCGAAGCCTGCACGGCGGACGCGCGGTGCCAGCG
TTTGCGCTCCGAGTATGTGGCGCAGTGCCTGGGCCGGGCTGCGCAGGGGGGCTGTCCCCGCG
CCCCTGCCGCGGGCCCTGCGCCGCTTCTTCGCCCCGCGGGCCGCCCGCGCTCACCCACGCA
CTGCTCTTCTGCCCCGTGCGCGGGCCCCGCGTGC**CGC**GAGCGTCGGCGCCAGACCTTCGTGCC
CTCCTGCGCCTTTTCGGGGCCCCGGCCCCGCGCCGCCCTCCTGCCTTGAGCCCTTAAACTTCT
GCGAGCGCAGCCGGGTCTGCAG**gtgcgcgcggggcggcgggcggggcgggctggcgagggtgggga**
cggggcctctctccgggtcacgcgcctccgcgcgcagGCCTCGCCTCCTGGCCTTTCAGG
TCTCGTGACCCCCAGCGCCAGCGCCCCGACGGCTGCCTGCTGGACCAGGGCGCCCGCTGC
CTGCGCGCTACGCGGGCCTCGTGG**gtacgcgcggggcgggatccggg**cgaggggcgggggttc
tccaggggatatctccgcgggtgggccgatgacttcgcgcctcagggtcccgagGCACC
GCCGTACCCCTAACTACGTGGACAACGTGAGCGCGCGCTGGCGCCCTGGTGCGACTGCGG
AGCCAGCGGAACCGCGGTGAGGACTGCGAAGCCTTCCGGGGGCTCTTTACCAGGAACCGCT
GCTTG**Ggtgaggggccccgggggggagtgagggggagtgggggcggcgcttactgccccctc**
ccaagccgcctgggtgggagccattttagagggggagaatggaagactgtacagttgagtcac
tctgtcacagctgtgcttattgttttgttattcctcaccacacaccttctgtccaaggagcc
agtctttgcagcaggggtctctcactttgtcccctgtgctgagccctgtgctaggggttcc
cagctaagtcacccctggacccctccctccatagATGGTGCCATTTCAGGCCTTTGCCAGCGG
GTGGCCCCCAGTCCTGCTGGACCAGCTGAACCCCCAGGGAGACCCGGAGCACAGCCTCCTGC
AG**gtaggtgcagggaggggaggggtgagctggcacctccccactgtcaccttcacacccttc**
cgtccctgggtgggcctgggtggaggcatgaagggcctgggggtgggggtgcaggcagagggca
gagacaggcttttgcctcaagtctgcacttgggtcccaccccccaagGTGTCTTCCACAGGCA
GGGCCCTGGAGAGACGCTCCCTGCTCTCCATACTTCTGTCTTGGCTCTCCCGGCCCTGCTC
TGATTAGGACAGCGACCTCGGATAGCACAGCCAGCTACTCCACCCTGCCTGCCTGGGCCGCC
TCTGTGGCCTACTGGCCCCCTTGAGAAGGGACTGGCTTACCCCCCAAGCCGGCCCTGGTGCTT
TCACTCCGCTGCCCTTTGTAGGTTTGGACACCCTGTGTGCCGTCCCCTGGGGCAAGGGATGT
AGGCTGGGGCCTGACTGTAAAGCCCCCGTCTCCCTGTCAGGAGGCATCTTGGTTGTAAGTCC
CTTTATTACAGACCTTGAGACCACTGGGGTCTCCACAAGGTGGGGTCAGGAGAGGTCACT
TTTGTAGCTGAGACCTCTCTGGAGACCCAGATCCCCTAGAGCAGGTCAGAGACATCCAGAAT
CCCAGAATTCTAGGAAATTGTATCAGCCTCCCAAGCATATAACCCCTAAGGAATCCATCGG
ACAAGACCCGTCTAACACTGCATCCTCCCAACTGGGGCATTACCCACCATTGTAGCCACCTG
TGCACCATGACATGCTGGGCAGAGTCTTTCCTGTTCCCCATATGCTGCCTGTGGAGGAAAAC
CTNGCAAGGGGCNA..

Fig. 24

SEQUENCE LISTING

<110> AIRAKSINEN, MATTI
SAARMA, MART

<120> COMPOUNDS RELATED TO OR DERIVED FROM GFRA4 AND THEIR
USE

<130> 9M34PC

<140>

<141>

<150> FI 20000394

<151> 2000-02-21

<160> 50

<170> PatentIn Ver. 2.1

<210> 1

<211> 260

<212> PRT

<213> Mus musculus

<220>

<223> GPI-linked isoform a1

<400> 1

Met Ala His Cys Met Glu Ser Ala Leu Leu Leu Leu Leu Leu Leu Gly
1 5 10 15

Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
20 25 30

Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu Tyr Val Ala
35 40 45

Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly Pro Gly Gly
50 55 60

Cys Val Arg Ser Arg Cys Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg
65 70 75 80

Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly Cys Glu Gly
85 90 95

Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro Ala Cys Ala

100 105 110
 Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu Pro Leu Glu
 115 120 125
 Arg Cys Glu Arg Ser Arg Leu Cys Arg Pro Arg Leu Leu Ala Phe Gln
 130 135 140
 Ala Ser Cys Ala Pro Ala Pro Gly Ser Arg Asp Arg Cys Pro Glu Glu
 145 150 155 160
 Gly Gly Pro Arg Cys Leu Arg Val Tyr Ala Gly Leu Ile Gly Thr Val
 165 170 175
 Val Thr Pro Asn Tyr Leu Asp Asn Val Ser Ala Arg Val Ala Pro Trp
 180 185 190
 Cys Gly Cys Ala Ala Ser Gly Asn Arg Arg Glu Glu Cys Glu Ala Phe
 195 200 205
 Arg Lys Leu Phe Thr Arg Asn Pro Cys Leu Asp Gly Ala Ile Gln Ala
 210 215 220
 Phe Asp Ser Leu Gln Pro Ser Val Leu Gln Asp Gln Thr Ala Gly Cys
 225 230 235 240
 Cys Phe Pro Arg Val Ser Trp Leu Tyr Ala Leu Thr Ala Leu Ala Leu
 245 250 255
 Gln Ala Leu Leu
 260

<210> 2

<211> 293

<212> PRT

<213> Mus musculus

<220>

<223> Putative transmembrane anchored isoform a2

<400> 2

Met Ala His Cys Met Glu Ser Ala Leu Leu Leu Leu Leu Leu Leu Gly
 1 5 10 15

Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
 20 25 30

Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu Tyr Val Ala
 35 40 45
 Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly Pro Gly Gly
 50 55 60
 Cys Val Arg Ser Arg Cys Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg
 65 70 75 80
 Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly Cys Glu Gly
 85 90 95
 Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro Ala Cys Ala
 100 105 110
 Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu Pro Leu Glu
 115 120 125
 Arg Cys Glu Arg Ser Arg Leu Cys Arg Pro Arg Leu Leu Ala Phe Gln
 130 135 140
 Ala Ser Cys Ala Pro Ala Pro Gly Ser Arg Asp Arg Cys Pro Glu Glu
 145 150 155 160
 Gly Gly Pro Arg Cys Leu Arg Val Tyr Ala Gly Leu Ile Gly Thr Val
 165 170 175
 Val Thr Pro Asn Tyr Leu Asp Asn Val Ser Ala Arg Val Ala Pro Trp
 180 185 190
 Cys Gly Cys Ala Ala Ser Gly Asn Arg Arg Glu Glu Cys Glu Ala Phe
 195 200 205
 Arg Lys Leu Phe Thr Arg Asn Pro Cys Leu Asp Gly Ala Ile Gln Ala
 210 215 220
 Phe Asp Ser Leu Gln Pro Ser Val Leu Gln Asp Gln Thr Ala Gly Cys
 225 230 235 240
 Cys Phe Pro Arg Ala Arg His Glu Trp Pro Glu Lys Ser Trp Arg Gln
 245 250 255
 Lys Gln Ser Leu Phe Cys Pro Asn Ala Gln Gly Val Leu Ala Val Cys
 260 265 270
 Thr His Cys Pro Gly Ser Pro Gly Pro Ala Leu Ile Arg Asn Met Asn
 275 280 285

Arg Gly Arg His Ser
290

<210> 3

<211> 190

<212> PRT

<213> Mus musculus

<220>

<223> Putative secreted isoform a3/4

<400> 3

Met Ala His Cys Met Glu Ser Ala Leu Leu Leu Leu Leu Leu Leu Gly
1 5 10 15

Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
20 25 30

Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu Tyr Val Ala
35 40 45

Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly Pro Gly Gly
50 55 60

Cys Val Arg Ser Arg Cys Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg
65 70 75 80

Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly Cys Glu Gly
85 90 95

Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro Ala Cys Ala
100 105 110

Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu Pro Leu Glu
115 120 125

Arg Cys Glu Arg Ser Arg Leu Cys Arg Val Cys Arg Ala Gly Arg Ala
130 135 140

Gly Pro Leu Thr Arg Val Arg Ala Arg Ala Gly Pro Val Ser Leu Pro
145 150 155 160

Ser Arg Pro His Ala Leu Pro Arg Pro Ala Pro Ala Thr Ala Ala Arg
165 170 175

Arg Arg Gly Ala Arg Val Val Cys Ala Ser Thr Gln Ala Ser
180 185 190

<210> 4

<211> 269

<212> PRT

<213> Homo sapiens

<220>

<223> GPI-anchored isoform a

<400> 4

Met Val Arg Cys Leu Gly Pro Ala Leu Leu Leu Leu Leu Leu Gly
 1 5 10 15

Ser Ala Ser Ser Val Gly Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
 20 25 30

Cys Thr Ala Asp Ala Arg Cys Gln Arg Leu Arg Ser Glu Tyr Val Ala
 35 40 45

Gln Cys Leu Gly Arg Ala Ala Gln Gly Gly Cys Pro Arg Ala Arg Cys
 50 55 60

Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg Gly Pro Pro Ala Leu Thr
 65 70 75 80

His Ala Leu Leu Phe Cys Pro Cys Ala Gly Pro Ala Cys Ala Glu Arg
 85 90 95

Arg Arg Gln Thr Phe Val Pro Ser Cys Ala Phe Ser Gly Pro Gly Pro
 100 105 110

Ala Pro Pro Ser Cys Leu Glu Pro Leu Asn Phe Cys Glu Arg Ser Arg
 115 120 125

Val Cys Arg Pro Arg Leu Leu Ala Phe Gln Val Ser Cys Thr Pro Ala
 130 135 140

Pro Ser Ala Pro Asp Gly Cys Leu Leu Asp Gln Gly Ala Arg Cys Leu
 145 150 155 160

Arg Ala Tyr Ala Gly Leu Val Gly Thr Ala Val Thr Pro Asn Tyr Val
 165 170 175

Asp Asn Val Ser Ala Arg Val Ala Pro Trp Cys Asp Cys Gly Ala Ser
 180 185 190

Gly Asn Arg Arg Glu Asp Cys Glu Ala Phe Arg Gly Leu Phe Thr Arg

195 200 205
 Asn Arg Cys Leu Asp Gly Ala Ile Gln Ala Phe Ala Ser Gly Trp Pro
 210 215 220
 Pro Val Leu Leu Asp Gln Leu Asn Pro Gln Gly Asp Pro Glu His Ser
 225 230 235 240
 Leu Leu Gln Val Ser Ser Thr Gly Arg Ala Leu Glu Arg Arg Ser Leu
 245 250 255
 Leu Ser Ile Leu Pro Val Leu Ala Leu Pro Ala Leu Leu
 260 265

<210> 5

<211> 299

<212> PRT

<213> Homo sapiens

<220>

<223> Putative GPI-anchored isoform b

<400> 5

Met Val Arg Cys Leu Gly Pro Ala Leu Leu Leu Leu Leu Leu Gly
 1 5 10 15
 Ser Ala Ser Ser Val Gly Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
 20 25 30
 Cys Thr Ala Asp Ala Arg Cys Gln Arg Leu Arg Ser Glu Tyr Val Ala
 35 40 45
 Gln Cys Leu Gly Arg Ala Ala Gln Gly Gly Cys Pro Arg Ala Arg Cys
 50 55 60
 Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg Gly Pro Pro Ala Leu Thr
 65 70 75 80
 His Ala Leu Leu Phe Cys Pro Cys Ala Gly Pro Ala Cys Ala Glu Arg
 85 90 95
 Arg Arg Gln Thr Phe Val Pro Ser Cys Ala Phe Ser Gly Pro Gly Pro
 100 105 110
 Ala Pro Pro Ser Cys Leu Glu Pro Leu Asn Phe Cys Glu Arg Ser Arg
 115 120 125

Val Cys Arg Cys Ala Arg Ala Ala Ala Gly Pro Trp Arg Gly Trp Gly
 130 135 140
 Arg Gly Leu Ser Pro Ala His Arg Pro Pro Ala Ala Gln Ala Ser Pro
 145 150 155 160
 Pro Gly Leu Ser Gly Leu Val His Pro Ser Ala Gln Arg Pro Arg Arg
 165 170 175
 Leu Pro Ala Gly Pro Gly Arg Pro Leu Pro Ala Arg Leu Arg Gly Pro
 180 185 190
 Arg Gly Val Pro Ala Gly Thr Ala Val Thr Pro Asn Tyr Val Asp Asn
 195 200 205
 Val Ser Ala Arg Val Ala Pro Trp Cys Asp Cys Gly Ala Ser Gly Asn
 210 215 220
 Arg Arg Glu Asp Cys Glu Ala Phe Arg Gly Leu Phe Thr Arg Asn Arg
 225 230 235 240
 Cys Leu Asp Gly Ala Ile Gln Ala Phe Ala Ser Gly Trp Pro Pro Val
 245 250 255
 Leu Leu Asp Gln Leu Asn Pro Gln Gly Asp Pro Glu His Ser Leu Leu
 260 265 270
 Gln Val Ser Ser Thr Gly Arg Ala Leu Glu Arg Arg Ser Leu Leu Ser
 275 280 285
 Ile Leu Pro Val Leu Ala Leu Pro Ala Leu Leu
 290 295

<210> 6

<211> 182

<212> PRT

<213> Homo sapiens

<220>

<223> Putative secreted isoform c

<400> 6

Met Val Arg Cys Leu Gly Pro Ala Leu Leu Leu Leu Leu Leu Gly
 1 5 10 15

Ser Ala Ser Ser Val Gly Gly Asn Arg Cys Val Asp Ala Ala Glu Ala
 20 25 30

Cys Thr Ala Asp Ala Arg Cys Gln Arg Leu Arg Ser Glu Tyr Val Ala
 35 40 45
 Gln Cys Leu Gly Arg Ala Ala Gln Gly Gly Cys Pro Arg Ala Arg Cys
 50 55 60
 Arg Arg Ala Leu Arg Arg Phe Phe Ala Arg Gly Pro Pro Ala Leu Thr
 65 70 75 80
 His Ala Leu Leu Phe Cys Pro Cys Ala Gly Pro Ala Cys Ala Glu Arg
 85 90 95
 Arg Arg Gln Thr Phe Val Pro Ser Cys Ala Phe Ser Gly Pro Gly Pro
 100 105 110
 Ala Pro Pro Ser Cys Leu Glu Pro Leu Asn Phe Cys Glu Arg Ser Arg
 115 120 125
 Val Cys Arg Pro Arg Leu Leu Ala Phe Gln Val Ser Cys Thr Pro Ala
 130 135 140
 Pro Ser Ala Pro Asp Gly Cys Leu Leu Asp Gln Gly Ala Arg Cys Leu
 145 150 155 160
 Arg Ala Tyr Ala Gly Leu Val Gly Ser Pro Gln Ala Pro Pro Ser Pro
 165 170 175
 Leu Thr Thr Trp Thr Thr
 180

<210> 7

<211> 783

<212> DNA

<213> Mus musculus

<220>

<223> Splice form (a1) for GPI linked protein

<400> 7

atggccact gcatggagtc tgcactgctg ctgttggtgc tgctgggggc tgcgagcttt 60
 accgacggga atcgctgcgt ggaacgggcc gaggcgtgta cagcagacga gcggtgccag 120
 cagctgcgct ctgagtacgt ggcaogatgc ctggggccggg cagcgcccg gggcaggccg 180
 ggacccgggg gctgcgtgcg ctcccgtgc cgcgagccc tgcgcccgtt cttcgcgcgt 240
 gggcctccgg cgctcacgca tgcgtgctc ttctgcggct gcgaaggctc cgcgtgcgcc 300
 gagcgccggc gccagacttt cgcgcccgc tgcgcgttct ccggcccggg gttggtgccg 360
 ccctcttgcc tggagcccct ggagcgctgc gagcgagcc gcctgtgccg gccccgtctc 420

```

cttgcccttcc aggcctcatg cgctcccgcg cccggctccc gcgaccgctg cccggaggag 480
gggggcccgc gttgtctgcg cgtctacgca ggcctcatag gcaccgtggt ccccccaac 540
tacctggaca acgtgagcgc gcgcgttgcg ccctggtgcg gctgtgcggc cagtggaaac 600
cggcgcggaag aatgcgaagc cttccgcaag ctctttacaa ggaaccctg cttggatggt 660
gccatacaag cctttgacag cttgcagcca tcagttctgc aggaccagac tgctgggtgc 720
tgtttcccgc ggggtgtcctg gctgtatgca ctactgccc tggctctcca ggccctgctc 780
tga 783

```

<210> 8

<211> 882

<212> DNA

<213> Mus musculus

<220>

<223> Splice form (a2) (for putative transmembrane
anchored protein)

<400> 8

```

atggccact gcatggagtc tgcaactgctg ctgttgttgc tgctggggtc tgogagcttt 60
accgacggga atcgctgcgt ggacgcggcc gaggcgtgta cagcagacga gcggtgccag 120
cagctgcgct ctgagtacgt ggcacgatgc ctgggccggg cagcgcccgg gggcaggccg 180
ggaccgggg gctgcgtgcg ctcccgtgcg cgccgagccc tgcgcgctt cttcgcgct 240
gggcctccg cgctcacgca tgcgctgctc ttctgaggct gcgaaggctc cgctgcgcc 300
gagcgccggc gccagacttt cgcgcccgcc tgcgcgttct ccggcccggg gttggtgccg 360
ccctcttgcc tggagccctt ggagcgctgc gagcgagcc gcctgtgccg gcccgtctc 420
cttgcccttcc aggcctcatg cgctcccgcg cccggctccc gcgaccgctg cccggaggag 480
gggggcccgc gttgtctgcg cgtctacgca ggcctcatag gcaccgtggt ccccccaac 540
tacctggaca acgtgagcgc gcgcgttgcg ccctggtgcg gctgtgcggc cagtggaaac 600
cggcgcggaag aatgcgaagc cttccgcaag ctctttacaa ggaaccctg cttggatggt 660
gccatacaag cctttgacag cttgcagcca tcagttctgc aggaccagac tgctgggtgc 720
tgtttcccgc gggcaaggca cgagtggcct gagaagagct ggaggcagaa acagtccttg 780
ttttgtccta acgccaagg tgctctggct gtatgcactc actgccctgg ctctccaggc 840
cctgctctga ttaggaacat gaaccgtgga cgacacagct ga 882

```

<210> 9

<211> 573

<212> DNA

<213> Mus musculus

<220>

<223> Splice form (a3/a4) (for secreted protein)

<400> 9

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atggccact gcatggagtc tgcaactgctg ctgttgttgc tgctggggtc tgogagcttt 60
accgacggga atcgctgcgt ggacgcggcc gaggcgtgta cagcagacga gcggtgccag 120
cagctgcgct ctgagtacgt ggcacgatgc ctgggccggg cagcgcccgg gggcaggccg 180
ggaccgggg gctgcgtgcg ctcccgtgcg cgccgagccc tgcgcgctt cttcgcgct 240
gggcctccg ctgcacgca tgcgctgctc ttctgaggct gcgaaggctc cgctgcgcc 300

```

```

gagcgccggc gccagacttt cgcgcccgcg tgcgcgttct ccggcccggg gttggtgccg 360
ccctcttgcc tggagccctt ggagcgctgc gagcgcagcc gcctgtgccg gtgcgtgcgt 420
gcggggcggg ctgggccgct caccgcgctc cgggcgcgcg caggccccgt ctccctgcct 480
tccaggcctc atgcgctccc gcgcccggct cccgcgaccg ctgcccgag gagggggggc 540
cgcgttgtct gcgcgtctac gcaggcctca tag 573

```

<210> 10

<211> 810

<212> DNA

<213> Homo sapiens

<220>

<223> Coding sequence of the splice form a for
GPI-anchored protein

<400> 10

```

atggtccgct gcctgggggc tgcgctgctg ctgctgctgt tactggggtc ggcgagctcg 60
gtcggagggg accgatgtgt ggacggggcc gaagcctgca cggcggacgc gcggtgccag 120
cgtttgcgct ccgagtatgt ggcgcagtgc ctgggccggg ctgcgcaggg gggctgtccc 180
cgcgcccgct gccgccgggc cctgcgcgcg ttcttcgccc gcgggccgcc cgcgctcacc 240
cacgcactgc tcttctgccc gtgcgcgggc cccgcgtgcg ccgagcgtcg gcgccagacc 300
ttcgtgccct cctgcgcctt ttccggggccc ggccccgcgc cgcctcctg ccttgagccc 360
ttaaacttct gcgagcgcag ccgggtctgc aggcctcgcc tcctggcctt tcaggctctg 420
tgcaccccag cggccagcgc ccccgacggc tgctgtctgg accagggcgc ccgctgcctg 480
cgcgcctacg cgggcctcgt gggcacggcc gtcaccccta actacgtgga caacgtgagc 540
gcgcgcgtgg cgccttggtg cgactgcgga gccagcggga accggcgtga ggactgcgaa 600
gccttcgggg ggctctttac caggaaccgc tgcttggtatg gtgccattca ggcctttgcc 660
agcgggtggc cccagtcctt gctggaccag ctgaaccccc agggagaccc ggagcacagc 720
ctcctgcagg tgtcctccac aggcaggggc ctggagagac gctccctgct ctccatactt 780
cctgtcctgg ctctcccgcc cctgctctga 810

```

<210> 11

<211> 900

<212> DNA

<213> Homo sapiens

<220>

<223> Splice form b

<400> 11

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atggtccgct gcctgggggc tgcgctgctg ctgctgctgt tactggggtc ggcgagctcg 60
gtcggagggg accgatgtgt ggacggggcc gaagcctgca cggcggacgc gcggtgccag 120
cgtttgcgct ccgagtatgt ggcgcagtgc ctgggccggg ctgcgcaggg gggctgtccc 180
cgcgcccgct gccgccgggc cctgcgcgcg ttcttcgccc gcgggccgcc cgcgctcacc 240
cacgcactgc tcttctgccc gtgcgcgggc cccgcgtgcg ccgagcgtcg gcgccagacc 300
ttcgtgccct cctgcgcctt ttccggggccc ggccccgcgc cgcctcctg ccttgagccc 360
ttaaacttct gcgagcgcag ccgggtctgc aggtgcgcgc gggcggcggc ggggccgtgg 420
cgagggtggg gacggggcct ctctccggct caccgccctc ccgccgcgca ggcctcgcct 480

```



```

cctggccttt caggtctcgt gcacccacgc gccagcgcc cccgacggct gcctgctgga 540
ccaggggcgcc cgctgcctgc ggcctacgc gggcctcgtg gggccccgc aggcacogcc 600
gtcaccccta actacgtgga caacgtgagc gcgcgcgtgg cgccctgggtg cgactgcgga 660
gccagcggga accggcgtga ggactgcgaa gccttccggg ggctctttac caggaaccgc 720
tgcttgatg gtgccattca ggcctttgcc agcgggtggc cccagtcct gctggaccag 780
ctgaaccccc agggagaccc ggagcacagc ctcttcagcagg tgcctccac aggcagggcc 840
ctggagagac gctccctgct ctccatactt cctgtcctgg ctctcccgcc cctgctctga 900

```

<210> 12

<211> 549

<212> DNA

<213> Homo sapiens

<220>

<223> Splice form c encoding putative secreted protein

<400> 12

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atggtccgct gcctggggcc tgcgctgctg ctgctgctgt tactgggggc ggcgagctcg 60
gtcggaggga accgatgtgt ggacgcggcc gaagcctgca cggcggacgc gcggtgccag 120
cgtttgcgct ccgagtatgt ggcgagtgcc ctggggccggg ctgcgcaggg gggctgtccc 180
cgcgcccgcct gccgcggggc cctgcgcgcg ttcttcgccc gcggggccgcc cgcgctcacc 240
cacgcactgc tcttctgccc gtgcgcgggc cccgcgtgcg ccgagcgtcg gcgccagacc 300
ttcgtgccct cctgcgcctt ttggggggcc ggccccgcgc cgccctcctg ccttgagccc 360
ttaaacttct gcgagcgcag cggggtctgc aggcctcgcc tcttggcctt tcaggtctcg 420
tgcaccccag cgcccagcgc ccccgacggc tgctgctgg accaggggcg ccgctgcctg 480
cgcgcctacg cgggcctcgt ggggtccccg caggcaccgc cgtcaccctt aactacgtgg 540
acaacgtga                                     549

```

<210> 13

<211> 2047

<212> DNA

<213> Homo sapiens

<220>

<221> intron

<222> (97)

<223> A genomic sequence containing an approximately 500 bp long intron between positions 97 and 98 (not shown)

<400> 13

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ctatcagact agggctctgc cagccatcct tctctgttga aggtccagca tgggtccgctg 60
cctggggcct gcgctgctgc tgctgctgtt actgggtagg gtcggcgagc tcggtcggag 120
ggaaccgatg tgtggacgcg gccgaagcct gcacggcgga cgcgcggtgc cagcgtttgc 180
gctccagata tgtggcgagc tgctggggcc gggctgcgca ggggggctgt ccccgcgccc 240
gctgccggcc ggccctgcgc cgcttcttgc cccgcggggc gcccgcgctc acccacgcac 300
tgctcttctg cccgtgcgcg ggccccgcgt gcgcccagcg tcggcgccag accttcgtgc 360
cctcctgcgc cttttcgggg cccggccccg cgcgcacctc ctgccttgag cccttaaaact 420

```

```

tctgcgagcg cagccgggtc tgcaggtgcg cgcgggcggc ggcggggccg tggcgagggt 480
ggggacgggg cctctctccg gctcaccgcc ctcccggcgc gcaggcctcg cctcctggcc 540
tttcaggtct cgtgcacccc agcgcccagc gcccccgacg gctgcctgct ggaccagggc 600
gcccgtgcc tgcgcgccta cgcgggcctc gtgggtacgc gcggccggga tccgggagag 660
ggcgggggtt ctccagggga tatctccgcc cgggtgggcc gatgacttcg ccctcagggt 720
ccccgcaggc accgccgtca cccctaacta cgtggacaac gtgagcgcgc gcgtggcgcc 780
ctggtgcgac tgcggagcca gcggaaccg gcgtgaggac tgcgaagcct tccgggggct 840
ctttaccagg aaccgctgct tgggtgaggg gcccgggggg gagtggaggg ggagtggggg 900
cggcgcttac tgccccctcc caagccgcct ggctgggagc catttttagag gggagaatgg 960
aagactgtac agttgagtca ctctgtcaca gctgtgctta ttgttttgtt attcctcacc 1020
acacaccttc tgtccaagga gccagtcctt gcagcagggg gtctctcact ttgtccctcg 1080
tgctgagccc tgtgctaggg tttcccagct aagtccaccc tggaccctc cctccataga 1140
tggtgccatt caggcctttg ccagcgggtg gccccagtc ctgctggacc agctgaaccc 1200
ccaggagagc ccggagcaca gcctcctgca ggtaggtgca gggaggggag ggtgagctgg 1260
cacctcccc actgtcacct tcacaccctt ccgtccctgg tgggcctggg tggaggcatg 1320
aagggcctgg ggtgggggtg caggcagagg gcagagacag gcttttgcct caagtctgca 1380
cttggtccc accccaagg tgtcctccac aggcagggcc ctggagagac gctccctgct 1440
ctccatactt cctgtcctgg ctctcccggc cctgctctga ttaggacagc gacctggat 1500
agcacagcca gctactccac cctgcctgcc tgggcgcct ctgtggccta ctggccctt 1560
gagaagggac tggcttaccc cccaagccgg ccctggtgct ttcactccgc tgccctttgt 1620
aggtttggac accctgtgtg ccgtccctg gggcaaggga ttaggctgg ggctgactg 1680
taaagcccc gtctccctgt caggaggcat cttggttga agtcccttta ttcacagacc 1740
ttgagaccac tggggtctcc cacaagggtg ggtcaggaga ggtcactttt gtagctgaga 1800
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aggaaattgt atcagcctcc caagcatata accccctaag gaatccatcg gacaagacc 1920
gtctaact gcatcctccc aactggggca ttaccacca ttgtagccac ctgtgcacca 1980
tgacatgctg ggcagagtct ttctgttcc ccatatgctg cctgtggagg aaaacctgca 2040
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<210> 14

<211> 24

<212> PRT

<213> Mus musculus

<220>

<223> Putative signaling sequence from alternative exon
1B

<400> 14

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Met Leu Arg Arg Ala His Leu Met Asp Glu Arg Pro Gly Gln Ala Ile
  1                   5                   10                   15

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Phe Leu Gly Leu Gly Ser Gln Arg
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<210> 15

<211> 53

<212> PRT

<213> Mus musculus

<220>

<223> Unique region of the secreted isoform (a3/4)

<400> 15

Cys Val Arg Ala Gly Arg Ala Gly Pro Leu Thr Arg Val Arg Ala Arg
 1 5 10 15

Ala Gly Pro Val Ser Leu Pro Ser Arg Pro His Ala Leu Pro Arg Pro
 20 25 30

Ala Pro Ala Thr Ala Ala Arg Arg Arg Gly Ala Arg Val Val Cys Ala
 35 40 45

Ser Thr Gln Ala Ser
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<210> 16

<211> 49

<212> PRT

<213> Mus musculus

<220>

<223> Putative transmembrane anchor sequence

<400> 16

Ala Arg His Glu Trp Pro Glu Lys Ser Trp Arg Gln Lys Gln Ser Leu
 1 5 10 15

Phe Cys Pro Asn Ala Gln Gly Val Leu Ala Val Cys Thr His Cys Pro
 20 25 30

Gly Ser Pro Gly Pro Ala Leu Ile Arg Asn Met Asn Arg Gly Arg His
 35 40 45

Ser

<210> 17

<211> 16

<212> PRT

<213> Mus musculus

<220>

<223> GPI anchor

<400> 17

Val Ser Trp Leu Tyr Ala Leu Thr Ala Leu Ala Leu Gln Ala Leu Leu
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<210> 18

<211> 23

<212> DNA

<213> Mus musculus

<220>

<223> 5' RACE primer 1

<400> 18

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<210> 19

<211> 20

<212> DNA

<213> Mus musculus

<220>

<223> 5' RACE primer 2

<400> 19

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<210> 20

<211> 20

<212> DNA

<213> Mus musculus

<220>

<223> 5' RACE primer 3

<400> 20

catcgtgccg cgtactcaga 20

<210> 21

<211> 24

<212> DNA

<213> Mus musculus

<220>

<223> 3' RACE primer 1

<400> 21
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<210> 22
<211> 23
<212> DNA
<213> Mus musculus

<220>
<223> 3' RACE primer 2

<400> 22
agagctggag gcagaaacag tcc 23

<210> 23
<211> 25
<212> DNA
<213> Mus musculus

<220>
<223> Exon 1a sense primer 1

<400> 23
ccaccatggc cactgcatg gagtc 25

<210> 24
<211> 20
<212> DNA
<213> Mus musculus

<220>
<223> Exon 1b sense primer 2

<400> 24
ccaccatggt gagaagagca 20

<210> 25
<211> 20
<212> DNA
<213> Mus musculus

<220>
<223> Exon 2 sense primer 3

<400> 25
gtgtacagca gacgagcggc 20

<210> 26
<211> 22
<212> DNA
<213> Mus musculus

<220>
<223> Exon 5 sense primer 5

<400> 26
atacaagcct ttgacagctt gc 22

<210> 27
<211> 21
<212> DNA
<213> Mus musculus

<220>
<223> Exon 4 antisense primer 4

<400> 27
gttccttgta aagagcttgc g 21

<210> 28
<211> 21
<212> DNA
<213> Mus musculus

<220>
<223> Exon 6 antisense primer 6

<400> 28
tggacaagat gcctactgac g 21

<210> 29
<211> 49
<212> DNA
<213> Homo sapiens

<220>
<223> Exon1 (5' UTR)

<400> 29
ctatcagact agggctctgc cagccatcct tctctgttga aggtccagc 49

<210> 30
<211> 46
<212> DNA
<213> Homo sapiens

<220>

<223> Exon1 (translated region)

<400> 30

atggtccgct gcctggggcc tgcgctgctg ctgctgctgt tactgg 46

<210> 31

<211> 346

<212> DNA

<213> Homo sapiens

<220>

<223> Exon2

<400> 31

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aggggggctg tccccgcgcc cgctgccgcc gggccctgcg ccgcttcttc gcccgcgggc 180
cgcccgcgct caccacgca ctgctcttct gccgctgcgc gggccccgcg tgcgccgagc 240
gtcggcgcca gaccttcgtg cctcctgcg ccttttcggg gcccgcccc gcgcgcacct 300
cctgccttga gcccttaaac ttctgcgagc gcagccgggt ctgcag 346

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<210> 32

<211> 79

<212> DNA

<213> Homo sapiens

<220>

<223> Intron 2

<400> 32

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<210> 33

<211> 110

<212> DNA

<213> Homo sapiens

<220>

<223> Exon3

<400> 33

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cctgctggac cagggcgccc gctgcctgcg cgctacgcg ggcctcgtgg 110

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<210> 34

<211> 94

<212> DNA

<213> Homo sapiens

<220>

<223> Intron3 (83 bp for alternative spliced putative
secreted form)

<400> 34

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tgggccgatg acttcgccct caggggtcccc gcag 94

<210> 35

<211> 135

<212> DNA

<213> Homo sapiens

<220>

<223> Exon4

<400> 35

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ggaaccgctg cttgg 135

<210> 36

<211> 276

<212> DNA

<213> Homo sapiens

<220>

<223> Intron4

<400> 36

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gccgcctggc tgggagccat tttagagggg agaatggaag actgtacagt tgagtcactc 120
tgtcacagct gtgcttattg ttttgttatt cctcaccaca caccttctgt ccaaggagcc 180
agtctttgca gcaggggggc tctcactttg tccctgtgc tgagccctgt gctagggttt 240
cccagctaag tccaccctgg acccctccct ccatag 276

<210> 37

<211> 92

<212> DNA

<213> Homo sapiens

<220>

<223> Exon5

<400> 37

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 cccagggaga cccggagcac agcctcctgc ag 92

<210> 38

<211> 168

<212> DNA

<213> Homo sapiens

<220>

<223> Intron5

<400> 38

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 cagagacagg cttttgcctc aagtctgcac ttggctccca cccccaag 168

<210> 39

<211> 81

<212> DNA

<213> Homo sapiens

<220>

<223> Exon6 (translated region)

<400> 39

gtgtcctcca caggcagggc cctggagaga cgctccctgc totccatact tcctgtcctg 60
 gctctcccg g cctgtctg a 81

<210> 40

<211> 557

<212> DNA

<213> Homo sapiens

<220>

<223> Exon6 (3' UTR)

<400> 40

ttaggacagc gacctcgat agcacagcca gctactccac cctgcctgcc tgggccgcct 60
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 ttcactccgc tgccctttgt aggtttggac accctgtgtg cgtcccttg gggcaaggga 180
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 ggtcactttt gtagctgaga cctctctgga gaccagatc ccctagagca ggtcagagac 360
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 gaatccatcg gacaagaccc gtctaacact gcctcctccc aactggggca ttaccacca 480
 ttgtagccac ctgtgcacca tgacatgctg ggcagagtct ttcctgttcc ccatatgctg 540
 cctgtggagg aaaacct 557

<210> 41

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

<400> 41

cacgttgtcc aggtagttgg

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<210> 42

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

<400> 42

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21

<210> 43

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

<400> 43

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21

<210> 44

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

<400> 44

gctcaccac gcactgctct tctg

24

<210> 45

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

<400> 45

cctaactacg tggacaacgt gagc

24

<210> 46

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

<400> 46

atggtgccat tcaggccttt gccag

25

<210> 47

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

<400> 47

gcaggtgtcc tccacaggca g

21

<210> 48

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

<400> 48

gaagtatgga gagcagggag cgtc

24

<210> 49

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

<400> 49

ccaccatggg ccgctgcctg g

21

<210> 50

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: -

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22

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 00/00994

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/71, A61K 38/17, C12N 5/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, A61K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nature, Volume 398, March 1999, Teresa M. Gunn et al, "The mouse mahogany locus encodes a transmembrane form of human attractin", page 152 - page 156, page 153, right column	1-15
A	--	16-18
X	Current Biology, Volume 8, 1998, Yasushi Enokido et al, "GFRalpha-4 and the tyrosine kinase Ret form a functional receptor complex for persephin" page 1019 - page 1022	1-15
A	--	16-18

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

4 May 2001

Date of mailing of the international search report

03 -07- 2001

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 00/00994

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Neuro Report, Volume 9, 1998, B. K. Dey et al, "Cloning of a novel murine isoform of the glial cell line-derived neurotrophic factor receptor" page 37 - page 42	1-15
A	--	16-18
E,X	WO 0102557 A1 (JANSSEN PHARMACEUTICA N.V.), 11 January 2001 (11.01.01), page 4, lines 26-30	1-15
P,X	Molecular and Cellular Neuroscience, Volume 15, 2000, Maria Lindahl et al, "Expression and Alternative Splicing of Mouse Gfra4 Suggest Roles in Endocrine Cell Development" page 522 - page 533	1-15
P,X	The Journal of Biological Chemistry, Volume 275, No 50, December 2000, Stefan Masure et al, "Mammalian GFRalpha-4, a Divergent Member of the GFRalpha Family of Coreceptors for Glial Cell Line-derived Neurotrophic Factor Family Ligands, Is a Receptor for the Neurotrophic Factor Persephin", page 39427 - page 39434	1-15
A	WO 9950298 A1 (MILLENNIUM PHARMACEUTICALS, INC.), 7 October 1999 (07.10.99), page 8, lines 3-15	1-18
X	WO 9962332 A1 (AIRAKSINEN, MATTI), 9 December 1999 (09.12.99)	16-18
P,X	EMBL/GenBank/DDBJ databases, Accession no. AL 356755, Sycamore N.: "Human DNA sequence from clone RP5-964F7 on chromosome 20 Contains part of a gene for a putative GDNF family receptor alpha 4 protein, the 3' part of a gene for a novel protein (disintegrin and metalloproteinase), ESTs, STSs, GSSs and CpG islands", 23 May 2000	1-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 00/00994

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EMBL/GenBank/DDBJ databases, Accession no. AF253318, Zhou B. et al: "Homo sapiens GFR receptor alpha 4 protein (GFRA4) mRNA, complete cds.", 26 October 2000 --	1-15
A	EMBL/GenBank/DDBJ databases, Accession no. AC17113, Waterston R.H: "Homo sapiens chromosome 20 clone RP11-574H7, Working Draft Sequence, 31 unordered pieces", 14 December 1999 --	1,3-9,11-15
A	EMBL/GenBank/DDBJ databases, Accession no. AC013324, Birren B. et al: "Homo sapiens chromosome 2 clone RP11-388K24 map 2, Low-pass sequence sampling", 9 November 1999 --	1,3-9,11-15
A	EMBL/GenBank/DDBJ databases, Accession no. AP002898, Yada T. et al: "Homo sapiens genomic DNA, chromosome 20 p, clone: 13N6, 25 October 2000 -- -----	1,3-9,11-15

INTERNATIONAL SEARCH REPORT

Int nal application No.
PCT/FI00/00994

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 18
because they relate to subject matter not required to be searched by this Authority, namely:
see next sheet*
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet**

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

*

Claim 18 relates to methods of treatment of the human or animal body by surgery or by therapy as well as diagnostic methods (Rule 39.1(iv)). Nevertheless, a search has been executed for this claim. The search has been based on the alleged effects of the compounds/compositions.

**

Unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features"-i.e. features that define a contribution which each of the inventions makes over the prior art (c.f. PCT Rule 13.2).

In view of the prior art in the present case such a unifying, novel technical feature has not been found between the different inventions revealed in the claims.

The prior art is represented by Gunn TM et al., Nature, Vol 398, 11 March 1999, pages 152-156, which discloses the localization of the mouse GFR α 4 gene. Thus, a mammalian GFR α 4 is known in the prior art and therefore it is considered to be obvious to a person skilled in the art to determine the cDNA for the receptor. No special technical feature has been shown to be present between the different sequences claimed. In the description to the present application it is stated that the mouse and human GFR α 4 lack the first Cys-rich domain common for all known GFR α 4-receptors (page 7, first paragraph). However, this discovery has not been shown to lead to any industrial application involving a unifying inventive concept.

Therefore, the international searching authority has arrived at the following principle of division:

Invention A, claims 1 (partly), 2, 4 (partly), 6-9 (partly), 10, 12-15 (partly), pertains to murine GFR α 4 corresponding to the sequences SEQ ID NOS: 1-3, 7-9.

Invention B, claims 1 (partly), 3, 4 (partly), 5, 6-9 (partly), 11, 12-15 (partly) pertains to human GFR α 4 corresponding to the sequences SEQ ID Nos: 4-6, 10-13, 29-39.

.../...

INTERNATIONAL SEARCH REPORT

Inter. application No.
PCT/FI00/00994

Invention C, claims 16-18, concerns a method for obtaining a cell-line useful in the production of a transgenic non-human animal. The method involves inactivating a functional GFR α 4 receptor. The method cannot be seen to involve any unifying special technical feature in common with mouse or human GFR α 4.

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/05/01

International application No.

PCT/FI 00/00994

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
WO	0102557	A1	11/01/01	AU	5810200	A	22/01/01
				GB	9915200	D	00/00/00
WO	9950298	A1	07/10/99	AU	3206899	A	18/10/99
WO	9962332	A1	09/12/99	AU	4618299	A	20/12/99
				FI	981217	A	30/11/99